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26129 15632 8041 25934 21263 12094 3514 30043 15134 24774 25723 29058
14294 10387 22923 13706 8226 14883 18231

Example 7. Selection of transgenic plants with enhanced agronomic trait(s)

This example illustrates identification of plant cells of the invention by screening derived plants and seeds for enhanced trait. Transgenic seed and plants in corn, soybean, cotton or canola with recombinant DNA constructs identified in Table 2 are prepared by plant cells transformed with DNA that is stably integrated into the genome of the corn cell. Progeny transgenic plants and seed of the transformed plant cells are screened for enhanced water use efficiency, enhanced cold tolerance, increased yield, enhanced nitrogen use efficiency, enhanced seed protein and enhanced seed oil as compared to control plants

A. Selection for enhanced Nitrogen Use Efficiency

Transgenic corn seeds provided by the present invention are planted in fields with three levels of nitrogen (N) fertilizer being applied, i.e. low level (0 N), medium level (80 lb/ac) and high level (180 lb/ac). A variety of physiological traits are monitored. Plants with enhanced NUE provide higher yield as compared to control plants.

B. Selection for increased yield

Effective selection of enhanced yielding transgenic plants uses hybrid progeny of the transgenic plants for corn, cotton, and canola, or inbred progeny of transgenic plants for soybeanplants plant such as corn, cotton, canola, or inbred plant such as soy, canola and cottoncotton over multiple locations with plants grown under optimal production management practices, and maximum pest control. A useful target for improved yield is a 5% to 10% increase in yield as compared to yield produced by plants grown from seed for a control plant. Selection methods may be applied in multiple and diverse geographic locations, for example up to 16 or more locations, over one or more planting seasons, for example at least two planting seasons, to statistically distinguish yield improvement from natural environmental effects.

In one field trial, transgenic corn plants comprising corn G1543 like 2 transgene as set forth in NUC SEQ ID NO: 74 under a tissue preferred promoter, such as green tissue promoter, have been shown to have an increased yield.

C. Selection for enhanced water use efficiency (WUE)

The selection process imposes a water withholding period to induce stressdrought followed by watering. For for example, for corn, a useful selection process imposes 3 drought/re-water cycles on plants over a total period of 15 days after an initial stress free growth period of 11 days. Each cycle consists of 5 days, with no water being applied for the first four days and a water quenching on the 5th day of the cycle. The primary phenotypes analyzed by the selection method are the changes in plant growth rate as determined by height and biomass during a vegetative drought treatment.

D. Selection for Growth Under Cold Stress

(1) Cold germination assay - Trays of transgenic and control seeds are placed in a growth chamber at 9.7°C for 24 days (no light). Seeds having higher germination rates as compared to the control are identified.

(2) Cold field efficacy trial - A cold field efficacy trial is used to identify gene constructs that confer enhanced cold vigor at germination and early seedling growth under early spring planting field conditions in conventional-till and simulated no-till environments. Seeds are planted into the ground around two weeks before local farmers begin to plant corn so that a significant cold stress is exerted onto the crop, named as cold treatment. Seeds also are planted under local optimal planting conditions such that the crop has little or no exposure to cold condition, named as normal treatment. At each location, seeds are planted under both cold and normal conditions with 3 repetitions per treatment. Two temperature monitors are set up at each location to monitor both air and soil temperature daily.

Seed emergence is defined as the point when the growing shoot breaks the soil surface. The number of emerged seedlings in each plot is counted everyday from the day the earliest plot begins to emerge until no significant changes in emergence occur. In addition, for each planting date, the latest date when emergence is 0 in all plots is also recorded. Seedling vigor is also rated at V3-V4 stage before the average of corn plant height reaches 10 inches, with 1=excellent early growth, 5=Average growth and 9=poor growth. Days to 50% emergence, maximum

percent emergence and seedling vigor are used to determine plants with enhanced cold tolerance.

E. Screens for transgenic plant seeds with increased protein and/or oil levels

This example sets forth a high-throughput selection for identifying plant seeds with improvement in seed composition using the Infratec 1200 series Grain Analyzer, which is a near-infrared transmittance spectrometer used to determine the composition of a bulk seed sample (Table 9). Near infrared analysis is a non-destructive, high-throughput method that can analyze multiple traits in a single sample scan. An NIR calibration for the analytes of interest is used to predict the values of an unknown sample. The NIR spectrum is obtained for the sample and compared to the calibration using a complex chemometric software package that provides predicted values as well as information on how well the sample fits in the calibration.

Infratec Model 1221, 1225, or 1227 with transport module by Foss North America is used with cuvette, item # 1000-4033, Foss North America or for small samples with small cell cuvette, Foss standard cuvette modified by Leon Girard Co. Corn and soy check samples of varying composition maintained in check cell cuvettes are supplied by Leon Girard Co. NIT collection software is provided by Maximum Consulting Inc.Software. Calculations are performed automatically by the software. Seed samples are received in packets or containers with barcode labels from the customer. The seed is poured into the cuvettes and analyzed as received.

Table 9.

Typical sample(s):	Whole grain corn and soybean seeds
Analytical time to run method:	Less than 0.75 min per sample
Total elapsed time per run:	1.5 minute per sample
Typical and minimum sample size:	Corn typical: 50cc; minimum 30cc Soybean typical: 50cc; minimum 5cc

Typical analytical range:	Determined in part by the specific calibration. Corn – moisture 5-15%, oil 5-20%, protein 5-30%, starch 50-75%, and density 1.0-1.3%. Soybean – moisture 5-15%, oil 15-25%, and protein 35-50%.

Example 8. Consensus sequence

This example illustrates the identification of consensus amino acid sequence for the proteins and homologs encoded by DNA that is used to prepare the transgenic seed and plants of this invention having enhanced agronomic traits.

ClustalW program was selected for multiple sequence alignments of the amino acid sequence of SEQ ID NO: 301 and its 20 homologs. Three major factors affecting the sequence alignments dramatically are (1) protein weight matrices; (2) gap open penalty; (3) gap extension penalty. Protein weight matrices available for ClustalW program include Blosum, Pam and Gonnet series. Those parameters with gap open penalty and gap extension penalty were extensively tested. On the basis of the test results, Blosum weight matrix, gap open penalty of 10 and gap extension penalty of 1 were chosen for multiple sequence alignment. Figure 1 shows the sequences of SEQ ID NO: 301, its homologs and the consensus sequence (SEQ ID NO: 30526) at the end. The symbols for consensus sequence are (1) uppercase letters for 100% identity in all positions of multiple sequence alignment output; (2) lowercase letters for >=70% identity; symbol; (3) "X" indicated <70% identity; (4) dashes "-" meaning that gaps were in >=70% sequences.

The consensus amino acid sequence can be used to identify DNA corresponding to the full scope of this invention that is useful in providing transgenic plants, for example corn and soybean plants with enhanced agronomic traits, for example improved nitrogen use efficiency, improved yield, improved water use efficiency and/or improved growth under cold stress, due to the expression in the plants of DNA encoding a protein with amino acid sequence identical to the consensus amino acid sequence.

Example 9. Identification of amino acid domain by Pfam analysis

This example illustrates the identification of domain and domain module by Pfam analysis.

The amino acid sequence of the expressed proteins that are shown to be associated with an enhanced trait are analyzed for Pfam protein family against the current Pfam collection of multiple sequence alignments and hidden Markov models using the HMMER. The Pfam protein domains and modules for the proteins of SEQ ID NO: 299 through 596 are shown in Tables 10, 11 and 12. The Hidden Markov model databases for the identified patent families allow identification of other homologous proteins and their cognate encoding DNA to enable the full breadth of the invention for a person of ordinary skill in the art. Certain proteins are identified by a single Pfam domain and others by multiple Pfam domains. For instance, the protein with amino acids of SEQ ID NO: 299 is characterized by three Pfam domains, i.e. KNOX1, KNOX2 and ELK.

Table 10. Pfam annotation

PEP SEQ ID NO	Gene ID	Pfam domain name	Begin	Stop	Score	E-value
299	PHE0007721_18998.pep	KNOX1	102	146	90.4	5.50E-24
299	PHE0007721_18998.pep	KNOX2	153	204	101.2	3.10E-27
299	PHE0007721_18998.pep	ELK	242	263	37	6.50E-08
300	PHE0007756_18009.pep	RPE65	105	591	864	7.30E-257
302	PHE0004988_15925.pep	F-box	4	52	23.7	0.00064
302	PHE0004988_15925.pep	LRR_1	157	179	9.7	4.4
302	PHE0004988_15925.pep	LRR_2	290	314	14.1	0.19
303	PHE0007140_21771.pep	MtN3_slv	9	98	98.2	2.50E-26
303	PHE0007140_21771.pep	MtN3_slv	134	221	73.6	6.20E-19
304	PHE0006823_16403.pep	Globin	10	149	102.6	1.10E-27
305	PHE0007440_22555.pep	Miro	14	128	88.4	2.20E-23
305	PHE0007440_22555.pep	Ras	14	175	332.3	8.40E-97
306	PHE0000206_22432.pep	Pkinase	79	337	342.2	8.80E-100
306	PHE0000206_22432.pep	efhand	384	412	25	0.00026
306	PHE0000206_22432.pep	efhand	456	484	24.9	0.00028
306	PHE0000206_22432.pep	efhand	490	518	35.1	2.40E-07
307	PHE0000598_16824.pep	HLH	385	434	54.6	3.30E-13
308	PHE0007590_17883.pep	S1	2	70	92.7	1.10E-24
309	PHE0007588_17881.pep	S1	2	86	73.5	6.50E-19
310	PHE0007592_17885.pep	S1	1	75	48	3.20E-11
311	PHE0007587_17880.pep	S1	3	75	106.6	7.10E-29
312	PHE0007624_17923.pep	S1	2	72	45.6	1.70E-10
312	PHE0007624_17923.pep	eIF-1a	5	70	134.9	2.20E-37
313	PHE0007591_17884.pep	S1	2	68	63.9	5.20E-16
314	PHE0007623_17922.pep	CN_hydrolase	11	184	166.3	7.50E-47

PEP SEQ ID NO	Gene ID	Pfam domain name	Begin	Stop	Score	E-value
315	PHE0007583_17871.pep	MtN3_slv	9	98	102.2	1.50E-27
315	PHE0007583_17871.pep	MtN3_slv	132	218	112.8	1.00E-30
316	PHE0006618_16146.pep	S10_plectin	3	98	240.6	3.40E-69
317	PHE0006791_16374.pep	GUN4	84	227	308.7	1.00E-89
318	PHE0007620_17918.pep	peroxidase	89	321	192.4	1.10E-54
319	PHE0006659_16195.pep	MFS_1	10	391	111.5	2.50E-30
321	PHE0006274_15867.pep	E2F_TDP	130	195	135.8	1.10E-37
322	PHE0006637_16166.pep	PP2C	7	305	255.6	1.00E-73
323	PHE0006926_16815.pep	VPS28	18	209	281.8	1.40E-81
324	PHE0006821_16402.pep	PALP	49	358	-65.8	0.00015
325	PHE0007687_18057.pep	p450	35	462	124	4.20E-34
326	PHE0007642_17999.pep	PAS	186	300	15.6	0.014
326	PHE0007642_17999.pep	PAS_3	211	303	25.3	0.00022
326	PHE0007642_17999.pep	PAS	464	578	17.8	0.0089
326	PHE0007642_17999.pep	PAS_3	489	581	23.5	0.00052
326	PHE0007642_17999.pep	Pkinase	663	952	269.7	5.80E-78
327	PHE0007677_18052.pep	FAD_binding_3	5	372	-96.1	4.10E-05
328	PHE0007644_18001.pep	LRRNT_2	23	64	49.2	1.40E-11
328	PHE0007644_18001.pep	LRR_1	97	119	10.5	3.1
328	PHE0007644_18001.pep	LRR_1	121	143	14.4	0.42
328	PHE0007644_18001.pep	LRR_1	145	167	12.1	1.5
328	PHE0007644_18001.pep	LRR_1	169	192	13	1.1
328	PHE0007644_18001.pep	LRR_1	194	216	14.1	0.52
328	PHE0007644_18001.pep	Pkinase	414	716	57.1	5.60E-14
329	PHE0007640_17992.pep	CorA	66	448	438.4	9.30E-129
330	PHE0007678_18053.pep	4HBT	66	151	56.4	9.40E-14
331	PHE0007649_18006.pep	Ank	108	140	50.4	6.20E-12
331	PHE0007649_18006.pep	Pkinase	196	455	113	8.80E-31
331	PHE0007649_18006.pep	Pkinase_Tyr	196	455	94.9	2.40E-25
332	PHE0006726_16298.pep	WD40	45	83	30.2	7.30E-06
332	PHE0006726_16298.pep	WD40	135	173	38.6	2.10E-08
333	PHE0006218_8776.pep	Pyr_redox_2	85	370	163.7	4.70E-46
333	PHE0006218_8776.pep	Pyr_redox	231	325	93.9	4.80E-25
333	PHE0006218_8776.pep	Thioredoxin	423	528	25	1.50E-06
333	PHE0006218_8776.pep	Glutaredoxin	446	508	27.6	4.30E-05
335	PHE0002554_17876.pep	Chloroa_b-bind	63	261	263.3	5.00E-76
336	PHE0006807_16388.pep	CCT	352	390	76	1.20E-19
337	PHE0003359_8487.pep	p450	51	489	120.5	4.90E-33
338	PHE0006542_15765.pep	PMSR	92	245	271	2.30E-78
339	PHE0007622_17921.pep	Spermine_synth	34	279	249.7	6.10E-72
340	PHE0007630_17956.pep	2OG-FeII_Oxy	190	291	149.7	7.90E-42
341	PHE0007593_17886.pep	Spermine_synth	28	273	503.6	2.20E-148
342	PHE0006475_15588.pep	PsbQ	3	162	209.5	7.60E-60
343	PHE0007619_17915.pep	MBD	11	84	65.4	1.80E-16
344	PHE0006846_16447.pep	Sterol_desat	1	186	76.3	9.30E-20
345	PHE0007584_17874.pep	MBD	4	77	81.3	3.00E-21

PEP SEQ ID NO	Gene ID	Pfam domain name	Begin	Stop	Score	E-value
346	PHE0003695_17913.pep	LRRNT_2	23	64	41.6	2.80E-09
346	PHE0003695_17913.pep	LRR_1	93	115	14.3	0.45
346	PHE0003695_17913.pep	LRR_1	117	139	18.3	0.028
346	PHE0003695_17913.pep	LRR_1	141	163	9.2	5.5
346	PHE0003695_17913.pep	LRR_1	165	186	12.7	1.2
346	PHE0003695_17913.pep	LRR_1	189	211	10.1	3.6
346	PHE0003695_17913.pep	LRR_1	213	235	9.8	4.3
346	PHE0003695_17913.pep	LRR_1	237	259	14.8	0.32
346	PHE0003695_17913.pep	LRR_1	260	281	10.6	2.9
346	PHE0003695_17913.pep	LRR_1	284	306	17.4	0.053
346	PHE0003695_17913.pep	LRR_1	308	330	13.8	0.65
346	PHE0003695_17913.pep	LRR_1	332	354	19.3	0.014
346	PHE0003695_17913.pep	LRR_1	356	378	9	5.8
346	PHE0003695_17913.pep	LRR_1	380	402	15.7	0.17
346	PHE0003695_17913.pep	LRR_1	404	426	11.8	1.8
346	PHE0003695_17913.pep	LRR_1	428	450	18.8	0.02
346	PHE0003695_17913.pep	LRR_1	452	474	13.2	0.93
346	PHE0003695_17913.pep	LRR_1	476	498	13.1	1
346	PHE0003695_17913.pep	LRR_1	500	521	10.4	3.3
346	PHE0003695_17913.pep	LRR_1	523	542	14.4	0.42
346	PHE0003695_17913.pep	Pkinase	648	917	111.8	2.00E-30
346	PHE0003695_17913.pep	Pkinase_Tyr	648	914	90.4	5.40E-24
347	PHE0003695_17879.pep	LRRNT_2	23	64	41.6	2.80E-09
347	PHE0003695_17879.pep	LRR_1	93	115	14.3	0.45
347	PHE0003695_17879.pep	LRR_1	117	139	18.3	0.028
347	PHE0003695_17879.pep	LRR_1	141	163	9.2	5.5
347	PHE0003695_17879.pep	LRR_1	165	186	12.7	1.2
347	PHE0003695_17879.pep	LRR_1	189	211	10.1	3.6
347	PHE0003695_17879.pep	LRR_1	213	235	9.8	4.3
347	PHE0003695_17879.pep	LRR_1	237	259	14.8	0.32
347	PHE0003695_17879.pep	LRR_1	260	281	10.6	2.9
347	PHE0003695_17879.pep	LRR_1	284	306	17.4	0.053
347	PHE0003695_17879.pep	LRR_1	308	330	13.8	0.65
347	PHE0003695_17879.pep	LRR_1	332	354	19.3	0.014
347	PHE0003695_17879.pep	LRR_1	356	378	9	5.8
347	PHE0003695_17879.pep	LRR_1	380	402	15.7	0.17
347	PHE0003695_17879.pep	LRR_1	404	426	11.8	1.8
347	PHE0003695_17879.pep	LRR_1	428	450	18.8	0.02
347	PHE0003695_17879.pep	LRR_1	452	474	13.2	0.93
347	PHE0003695_17879.pep	LRR_1	476	498	13.1	1
347	PHE0003695_17879.pep	LRR_1	500	521	10.4	3.3
347	PHE0003695_17879.pep	LRR_1	523	542	14.4	0.42
347	PHE0003695_17879.pep	Pkinase	648	917	111.8	2.00E-30
347	PHE0003695_17879.pep	Pkinase_Tyr	648	914	90.4	5.40E-24
349	PHE0007645_18002.pep	AP2	115	180	156.4	7.20E-44
350	PHE0006784_16366.pep	PALP	12	301	453.6	2.50E-133

PEP SEQ ID NO	Gene ID	Pfam domain name	Begin	Stop	Score	E-value
351	PHE0006171_16491.pep	Glyoxalase	70	214	136.8	5.90E-38
353	PHE0006906_16796.pep	Proteasome	10	206	145.9	1.00E-40
354	PHE0006649_16180.pep	AP2	46	110	149.5	8.60E-42
355	PHE0006809_16390.pep	Prismane	1	548	886.8	9.80E-264
356	PHE0006918_16808.pep	SWIM	321	353	42.8	1.10E-09
357	PHE0007813_18219.pep	MtN3_slv	9	98	79.8	8.50E-21
357	PHE0007813_18219.pep	MtN3_slv	132	218	125.3	1.80E-34
359	PHE0006825_16405.pep	Cytochrom_C552	27	476	1333.6	0
360	PHE0006964_16864.pep	tRNA_anti	43	122	55.2	2.10E-13
360	PHE0006964_16864.pep	tRNA-synt_2	140	557	208.2	1.90E-59
361	PHE0008105_18407.pep	Histone	152	225	69.9	8.40E-18
361	PHE0008105_18407.pep	CBFD_NFYB_HMF	158	222	77.9	3.10E-20
362	PHE0006630_16159.pep	Ldh_1_N	90	239	202	1.30E-57
362	PHE0006630_16159.pep	Ldh_1_C	241	413	258.1	1.80E-74
363	PHE0006966_16868.pep	DNA_photolyase	12	183	298.7	1.10E-86
363	PHE0006966_16868.pep	FAD_binding_7	214	492	536.3	3.30E-158
365	PHE0006608_16123.pep	GSHPx	42	150	237.8	2.30E-68
366	PHE0010462_21459.pep	NUDIX	17	159	111.7	2.20E-30
366	PHE0010462_21459.pep	NUDIX	190	322	100.5	4.90E-27
367	PHE0009211_21774.pep	Auxin_inducible	1	106	149.5	8.80E-42
368	PHE0006256_8775.pep	RPE65	105	591	864.5	5.10E-257
369	PHE0006989_16918.pep	Pkinase	88	396	224.8	1.90E-64
370	PHE0007649_18166.pep	Ank	108	140	50.4	6.20E-12
370	PHE0007649_18166.pep	Pkinase	196	455	115.1	2.00E-31
370	PHE0007649_18166.pep	Pkinase_Tyr	196	455	98.9	1.50E-26
371	PHE0006708_16264.pep	DEAD	57	223	202.7	8.40E-58
371	PHE0006708_16264.pep	Helicase_C	291	367	127.8	3.10E-35
372	PHE0001117_19094.pep	Homeobox	79	133	68.2	2.70E-17
372	PHE0001117_19094.pep	HALZ	134	178	66.7	7.20E-17
373	PHE0006266_8814.pep	Fe_bilin_red	16	244	209.5	7.50E-60
374	PHE0006795_16378.pep	Na_H_antiport_1	4	380	875.9	1.90E-260
375	PHE0006956_16853.pep	Molybdop_Fe4S4	2	56	92	1.90E-24
375	PHE0006956_16853.pep	Molybdopterin	59	482	377.8	1.60E-110
375	PHE0006956_16853.pep	Molydop_binding	578	689	159.8	7.10E-45
375	PHE0006956_16853.pep	Fer2_BFD	825	877	62	2.00E-15
377	PHE0006824_16404.pep	Globin	14	154	93.4	7.00E-25
378	PHE0006812_16393.pep	Bac_globin	3	118	188.4	1.70E-53
379	PHE0006815_16396.pep	Bac_globin	26	146	191.4	2.10E-54
380	PHE0006806_16387.pep	Bac_globin	26	146	196.8	5.10E-56
381	PHE0006624_16153.pep	NAD_binding_2	3	178	282.5	7.90E-82
381	PHE0006624_16153.pep	6PGD	182	473	713.8	1.20E-211
382	PHE0005006_15823.pep	Chloroa_b-bind	63	242	241.4	1.90E-69
384	PHE0006683_16229.pep	Response_reg	10	151	73.8	5.40E-19
385	PHE0006884_16703.pep	Glyco_transf_5	83	344	462.7	4.80E-136
385	PHE0006884_16703.pep	Glycos_transf_1	384	567	40.2	7.10E-09
386	PHE0006691_16237.pep	NAD_Gly3P_dh_N	6	165	285	1.40E-82

PEP SEQ ID NO	Gene ID	Pfam domain name	Begin	Stop	Score	E-value
386	PHE0006691_16237.pep	ApbA	8	169	6.6	0.00011
386	PHE0006691_16237.pep	NAD_Gly3P_dh_C	183	327	289.1	8.20E-84
387	PHE0006790_16372.pep	Rieske	220	317	96.7	7.10E-26
387	PHE0006790_16372.pep	PaO	408	507	180.7	3.60E-51
388	PHE0009143_19932.pep	Histone_HNS	21	129	170.1	5.70E-48
389	PHE0008406_18830.pep	AA_permease	57	511	619.8	2.40E-183
390	PHE0008279_18708.pep	Mit_rib_S27	14	93	135.3	1.70E-37
391	PHE0009142_19931.pep	Histone_HNS	21	130	175.1	1.70E-49
393	PHE0007639_17991.pep	ELFV_dehydrog_N	84	214	298.9	9.20E-87
393	PHE0007639_17991.pep	ELFV_dehydrog	229	474	469.7	3.60E-138
394	PHE0007646_18170.pep	F-box	36	83	51.8	2.20E-12
394	PHE0007646_18170.pep	Kelch_1	131	185	25.1	0.00026
394	PHE0007646_18170.pep	Kelch_1	187	233	44.2	4.40E-10
394	PHE0007646_18170.pep	Kelch_2	187	233	26.1	0.00012
395	PHE0007765_18182.pep	GST_N	5	79	69.6	1.00E-17
395	PHE0007765_18182.pep	GST_C	101	204	28.8	1.90E-05
396	PHE0008167_18465.pep	Pkinase	8	262	210	5.60E-60
396	PHE0008167_18465.pep	Pkinase_Tyr	8	262	247.9	2.10E-71
398	PHE0008183_18479.pep	LRRNT_2	23	66	49.3	1.30E-11
398	PHE0008183_18479.pep	LRR_1	71	93	12.1	1.6
398	PHE0008183_18479.pep	LRR_1	95	117	10.3	3.3
398	PHE0008183_18479.pep	LRR_1	119	142	13	1
398	PHE0008183_18479.pep	LRR_1	144	166	19.5	0.012
398	PHE0008183_18479.pep	LRR_1	168	190	10.6	3
398	PHE0008183_18479.pep	LRR_1	192	214	8.8	6.4
398	PHE0008183_18479.pep	LRR_1	289	311	17.3	0.055
398	PHE0008183_18479.pep	LRR_1	313	335	10.6	2.9
398	PHE0008183_18479.pep	LRR_1	337	359	10.8	2.8
398	PHE0008183_18479.pep	LRR_1	361	384	12	1.6
398	PHE0008183_18479.pep	LRR_1	409	431	10.4	3.2
398	PHE0008183_18479.pep	LRR_1	457	479	11.9	1.7
398	PHE0008183_18479.pep	LRR_1	481	503	10.2	3.4
398	PHE0008183_18479.pep	LRR_1	505	527	10.4	3.3
398	PHE0008183_18479.pep	LRR_1	529	551	10.9	2.6
398	PHE0008183_18479.pep	LRR_1	553	575	9.3	5.2
398	PHE0008183_18479.pep	LRR_1	577	598	11	2.4
398	PHE0008183_18479.pep	Pkinase	695	966	133.8	4.80E-37
398	PHE0008183_18479.pep	Pkinase_Tyr	695	966	134.8	2.30E-37
399	PHE0007585_17875.pep	LEA_3	2	98	138.9	1.30E-38
400	PHE0006804_16385.pep	zf-CCCH	13	39	35.4	1.90E-07
400	PHE0006804_16385.pep	zf-CCCH	149	174	24.3	0.00045
401	PHE0006703_15751.pep	PFK	72	347	650.2	1.70E-192
402	PHE0007410_17653.pep	Acyltransferase	27	249	29.2	1.00E-05
403	PHE0006854_16456.pep	YABBY	40	278	249.1	9.30E-72
404	PHE0007721_21293.pep	KNOX1	102	146	90.4	5.50E-24
404	PHE0007721_21293.pep	KNOX2	153	204	101.2	3.10E-27

PEP SEQ ID NO	Gene ID	Pfam domain name	Begin	Stop	Score	E-value
404	PHE0007721_21293.pep	ELK	242	263	37	6.50E-08
405	PHE0006563_15990.pep	GATase_2	2	161	103.9	4.70E-28
405	PHE0006563_15990.pep	Asn_synthase	209	450	328.7	9.80E-96
406	PHE0006619_16147.pep	AA_kinase	15	261	192.3	1.20E-54
407	PHE0006813_16394.pep	Bac_globin	7	124	123	8.50E-34
408	PHE0006688_16234.pep	Ribosomal_S2	13	229	448.6	8.00E-132
409	PHE0006635_16164.pep	MGS	28	121	99.4	1.10E-26
411	PHE0008416_18838.pep	PTR2	115	504	350.4	3.00E-102
413	PHE0008599_19166.pep	Isoamylase_N	24	115	93.4	6.60E-25
413	PHE0008599_19166.pep	Alpha-amylase	164	578	75.8	1.40E-19
414	PHE0008375_18732.pep	Pribosyltran	93	237	141.8	1.80E-39
416	PHE0008394_18763.pep	Gln-synt_N	68	148	147.3	4.20E-41
416	PHE0008394_18763.pep	Gln-synt_C	154	406	287.7	2.20E-83
417	PHE0008395_18764.pep	Gln-synt_N	68	148	147.3	4.20E-41
417	PHE0008395_18764.pep	Gln-synt_C	154	406	285	1.50E-82
418	PHE0008444_18846.pep	Gln-synt_N	17	97	150.4	4.70E-42
418	PHE0008444_18846.pep	Gln-synt_C	103	355	283.9	3.00E-82
419	PHE0006819_16400.pep	Lactamase_B	47	236	77.1	5.60E-20
419	PHE0006819_16400.pep	Flavodoxin_1	267	397	8.1	0.0041
419	PHE0006819_16400.pep	Flavin_Reduct	424	573	254.7	1.90E-73
420	PHE0007417_17663.pep	EIN3	60	467	777.5	7.80E-231
421	PHE0008443_18845.pep	GATase_2	2	161	98.7	1.70E-26
421	PHE0008443_18845.pep	Asn_synthase	209	450	331	2.00E-96
422	PHE0006818_16399.pep	Lactamase_B	34	227	105.3	1.80E-28
422	PHE0006818_16399.pep	Flavodoxin_1	256	389	86.4	8.80E-23
422	PHE0006818_16399.pep	Rubredoxin	423	471	89.8	8.10E-24
423	PHE0006579_16031.pep	Thi4	63	306	552.2	5.40E-163
423	PHE0006579_16031.pep	DAO	91	319	-9	1.90E-05
424	PHE0006808_16389.pep	CCT	354	392	75.3	1.90E-19
425	PHE0008160_18460.pep	TPP_enzyme_N	45	221	286.5	5.20E-83
425	PHE0008160_18460.pep	TPP_enzyme_M	243	376	82.1	1.70E-21
425	PHE0008160_18460.pep	TPP_enzyme_C	431	578	34.8	2.80E-09
426	PHE0007571_17834.pep	GSH_synth_ATP	29	499	836.5	1.30E-248
426	PHE0007571_17834.pep	GSH_synthase	229	331	200	5.50E-57
427	PHE0007586_17877.pep	Chloroa_b-bind	64	261	255.3	1.20E-73
429	PHE0009729_21717.pep	Homeobox	67	123	68.9	1.60E-17
429	PHE0009729_21717.pep	HALZ	124	168	53.4	7.60E-13
430	PHE0006424_15520.pep	PsbW_2	1	133	382.1	8.60E-112
431	PHE0006392_15480.pep	PsbP	106	322	252.9	6.50E-73
432	PHE0006589_16093.pep	Thioredoxin	64	167	130.3	5.20E-36
433	PHE0009258_21487.pep	Na_H_Exchanger	22	441	181.7	1.70E-51
434	PHE0006680_16226.pep	2OG-FeII_Oxy	187	287	158	2.40E-44
435	PHE0006481_16072.pep	adh_short	33	181	28.8	7.00E-08
436	PHE0006269_8820.pep	TPR_1	74	107	21.3	0.0035
436	PHE0006269_8820.pep	TPR_2	74	107	24.7	0.00034
436	PHE0006269_8820.pep	TPR_1	108	141	37.4	4.80E-08

PEP SEQ ID NO	Gene ID	Pfam domain name	Begin	Stop	Score	E-value
436	PHE0006269_8820.pep	TPR_2	108	141	35.3	2.10E-07
436	PHE0006269_8820.pep	TPR_1	142	175	15	0.086
436	PHE0006269_8820.pep	TPR_2	142	175	23.8	0.00063
436	PHE0006269_8820.pep	TPR_1	179	212	7.9	0.61
436	PHE0006269_8820.pep	TPR_4	247	272	23.6	0.00069
436	PHE0006269_8820.pep	TPR_1	281	314	16.2	0.063
436	PHE0006269_8820.pep	TPR_2	281	314	26	0.00014
436	PHE0006269_8820.pep	TPR_1	349	382	13.6	0.13
437	PHE0006783_16365.pep	Aa_trans	32	469	574.9	7.90E-170
438	PHE0003151_18392.pep	AP2	318	391	149.5	8.70E-42
438	PHE0003151_18392.pep	AP2	420	485	125.8	1.20E-34
439	PHE0008272_18723.pep	HSF_DNA-bind	18	188	213.9	3.50E-61
440	PHE0008277_18706.pep	SNF5	172	400	471.8	8.60E-139
441	PHE0008109_18411.pep	Sad1_UNC	203	330	195.6	1.10E-55
442	PHE0008280_18826.pep	Alpha-amylase	14	452	198.9	1.20E-56
443	PHE0002424_15825.pep	adh_short	43	215	24.9	1.30E-07
443	PHE0002424_15825.pep	KR	43	200	-69.1	0.0024
444	PHE0006914_16804.pep	AAA	204	391	298.6	1.10E-86
445	PHE0006642_16172.pep	p450	42	496	213.5	4.80E-61
447	PHE0009926_21079.pep	DSPc	24	152	137.5	3.60E-38
448	PHE0006206_19159.pep	Sina	106	305	426.4	4.00E-125
449	PHE0008274_18705.pep	BURP	56	280	380	3.60E-111
450	PHE0009510_20526.pep	Spermine_synth	38	283	500.4	2.10E-147
451	PHE0009937_21097.pep	Spermine_synth	38	283	500.4	2.10E-147
452	PHE0009927_21080.pep	DSPc	24	162	146.6	6.60E-41
454	PHE0006445_15956.pep	Allene_ox_cyc	74	253	401.6	1.20E-117
455	PHE0006987_16893.pep	efhand	86	114	25	0.00026
455	PHE0006987_16893.pep	efhand	167	195	27.6	4.30E-05
456	PHE0004230_15865.pep	E2F_TDP	146	211	133.8	4.60E-37
457	PHE0006814_16395.pep	Bac_globin	26	144	206	8.80E-59
458	PHE0002773_15881.pep	NTP_transferase	88	366	415.4	8.30E-122
459	PHE0006060_15842.pep	ubiquitin	5	73	138	2.50E-38
459	PHE0006060_15842.pep	ubiquitin	81	149	138	2.50E-38
459	PHE0006060_15842.pep	ubiquitin	157	225	138	2.50E-38
459	PHE0006060_15842.pep	ubiquitin	233	301	138	2.50E-38
460	PHE0006850_16451.pep	YABBY	6	166	342.9	5.20E-100
461	PHE0008043_18199.pep	Homeobox	25	85	64.6	3.20E-16
461	PHE0008043_18199.pep	START	164	374	198.3	1.80E-56
461	PHE0008043_18199.pep	MEKHLA	693	840	296.7	4.40E-86
462	PHE0000125_18852.pep	Response_reg	28	153	25.4	3.80E-05
462	PHE0000125_18852.pep	CCT	457	495	70.6	5.10E-18
463	PHE0008269_18720.pep	Pkinase	23	279	234	3.30E-67
464	PHE0008264_18712.pep	zf-UBR	40	109	73.4	7.10E-19
465	PHE0002782_20059.pep	PGI	2	457	172.8	8.80E-49
466	PHE0009438_20404.pep	GATA	191	226	72.7	1.10E-18
467	PHE0009429_20392.pep	ParBc	70	151	47.5	4.50E-11

PEP SEQ ID NO	Gene ID	Pfam domain name	Begin	Stop	Score	E-value
468	PHE0009440_20406.pep	ParBc	11	126	55.3	2.00E-13
469	PHE0008543_18945.pep	TP_methylase	222	435	272.6	7.70E-79
470	PHE0006960_16857.pep	Oxidored_molyb	116	301	289.2	7.70E-84
470	PHE0006960_16857.pep	Mo-co_dimer	326	459	300.9	2.30E-87
470	PHE0006960_16857.pep	Cyt-b5	515	588	93.3	7.20E-25
470	PHE0006960_16857.pep	FAD_binding_6	633	740	212.6	9.20E-61
470	PHE0006960_16857.pep	NAD_binding_1	759	872	169.6	8.00E-48
471	PHE0009427_20389.pep	IGPD	103	247	372.6	6.20E-109
472	PHE0006437_15906.pep	Pkinase	39	307	15.8	1.70E-09
473	PHE0007409_17652.pep	DAGAT	64	340	278.2	1.60E-80
474	PHE0009426_20387.pep	PHP	2	281	104.4	3.30E-28
475	PHE0009430_20393.pep	ParBc	44	128	45.7	1.60E-10
476	PHE0007003_16906.pep	PTPA	96	396	524	1.60E-154
477	PHE0008378_18741.pep	bZIP_1	223	280	76.5	8.20E-20
477	PHE0008378_18741.pep	bZIP_2	224	277	47.5	4.40E-11
478	PHE0008273_18725.pep	zf-CCCH	80	106	39.3	1.30E-08
479	PHE0008547_18953.pep	Fer2	20	95	95	2.20E-25
480	PHE0006922_16812.pep	Hexokinase_1	44	251	260.7	2.90E-75
480	PHE0006922_16812.pep	Hexokinase_2	258	501	261.2	2.10E-75
481	PHE0006920_16810.pep	AAA	267	458	270	4.60E-78
481	PHE0006920_16810.pep	Vps4_C	464	514	4.5	0.00031
482	PHE0008396_18765.pep	Gln-synt_N	68	148	147.3	4.20E-41
482	PHE0008396_18765.pep	Gln-synt_C	154	406	285.4	1.10E-82
483	PHE0006957_16854.pep	Gln-synt_N	68	148	147.3	4.20E-41
483	PHE0006957_16854.pep	Gln-synt_C	154	406	285.4	1.10E-82
484	PHE0006404_15936.pep	RRM_1	8	77	81.4	2.80E-21
484	PHE0006404_15936.pep	RRM_1	112	182	81.8	2.10E-21
485	PHE0006820_16401.pep	Lactamase_B	47	236	82.8	1.10E-21
485	PHE0006820_16401.pep	Flavin_Reduct	423	572	222.8	7.90E-64
486	PHE0006690_16236.pep	MFS_1	57	421	44.3	4.30E-10
487	PHE0006395_15932.pep	LRR_1	185	206	11.9	1.7
487	PHE0006395_15932.pep	LRR_1	208	229	17.1	0.065
487	PHE0006395_15932.pep	LRR_1	231	252	13.5	0.77
487	PHE0006395_15932.pep	LRR_1	254	272	12.6	1.2
487	PHE0006395_15932.pep	LRR_1	278	299	8.4	7.8
487	PHE0006395_15932.pep	LRR_1	349	370	20.8	0.0049
487	PHE0006395_15932.pep	LRR_1	372	394	7.8	9.8
488	PHE0006759_16384.pep	SHMT	55	453	1032.6	0
489	PHE0006576_16028.pep	Myb_DNA-binding	11	57	59.3	1.30E-14
489	PHE0006576_16028.pep	Myb_DNA-binding	63	108	48.9	1.70E-11
490	PHE0006979_16885.pep	Tryp_alpha_amyl	33	111	55.3	2.00E-13
491	PHE0009786_21754.pep	HLH	33	80	30	8.20E-06
492	PHE0006572_16007.pep	Pkinase	11	269	317	3.30E-92
494	PHE0006588_16092.pep	Thioredoxin	56	161	128.8	1.50E-35
495	PHE0006985_16891.pep	DUF1716	13	118	159.3	1.00E-44
496	PHE0006875_16688.pep	PSI_PSAK	44	138	191.6	1.90E-54

PEP SEQ ID NO	Gene ID	Pfam domain name	Begin	Stop	Score	E-value
497	PHE0006626_16155.pep	Glyco_hydro_17	30	335	587.5	1.20E-173
498	PHE0006689_16235.pep	Aa_trans	165	548	224.1	3.20E-64
499	PHE0006799_16382.pep	B12-binding	10	135	104.3	3.70E-28
499	PHE0006799_16382.pep	Radical_SAM	202	368	91.2	3.10E-24
500	PHE0006959_16856.pep	Oxidored_molyb	143	328	286.7	4.40E-83
500	PHE0006959_16856.pep	Mo-co_dimer	353	486	297.6	2.40E-86
500	PHE0006959_16856.pep	Cyt-b5	543	616	90.4	5.60E-24
500	PHE0006959_16856.pep	FAD_binding_6	660	767	231.6	1.70E-66
500	PHE0006959_16856.pep	NAD_binding_1	786	899	186.4	7.00E-53
501	PHE0011615_23861.pep	Pkinase	267	523	297.5	2.50E-86
501	PHE0011615_23861.pep	Pkinase_Tyr	267	523	127.5	3.70E-35
502	PHE0006925_16814.pep	Tim17	4	133	198.1	2.10E-56
503	PHE0006713_16274.pep	APC8	1	161	401.5	1.20E-117
503	PHE0006713_16274.pep	TPR_1	339	372	34.7	3.20E-07
503	PHE0006713_16274.pep	TPR_2	339	372	23.7	0.00066
503	PHE0006713_16274.pep	TPR_2	373	406	23.7	0.00068
503	PHE0006713_16274.pep	TPR_1	373	406	34.1	4.80E-07
503	PHE0006713_16274.pep	TPR_2	407	440	22.1	0.002
503	PHE0006713_16274.pep	TPR_1	407	440	24.3	0.00043
504	PHE0008447_18848.pep	Lir1	1	126	178.6	1.60E-50
505	PHE0006674_16221.pep	PTR2	96	505	222.7	8.20E-64
506	PHE0010613_22398.pep	DAGK_cat	43	184	106.4	8.50E-29
507	PHE0002720_22558.pep	DnaJ	90	151	137.6	3.40E-38
507	PHE0002720_22558.pep	DnaJ_CXXCXGXG	218	301	100.5	5.20E-27
507	PHE0002720_22558.pep	DnaJ_C	314	436	210.6	3.60E-60
508	PHE0011760_24005.pep	Pkinase	400	656	297.5	2.50E-86
508	PHE0011760_24005.pep	Pkinase_Tyr	400	656	127.5	3.70E-35
509	PHE0010612_22397.pep	G-patch	15	59	62	1.90E-15
511	PHE0007411_17654.pep	Acyltransferase	136	283	86.4	8.50E-23
512	PHE0010617_22402.pep	Isochorismatase	27	210	-41	0.0027
513	PHE0010610_22395.pep	zf-C3HC4	103	144	36.3	1.10E-07
514	PHE0006883_16702.pep	Glyco_transf_5	78	339	461.4	1.10E-135
514	PHE0006883_16702.pep	Glycos_transf_1	379	562	40.5	5.80E-09
515	PHE0010636_22408.pep	UAA	96	387	-125.2	0.00037
515	PHE0010636_22408.pep	DUF6	105	230	32.1	2.00E-06
515	PHE0010636_22408.pep	TPT	239	384	188.8	1.30E-53
516	PHE0011082_23038.pep	adh_short	19	187	111.5	2.50E-30
516	PHE0011082_23038.pep	KR	19	202	-67.5	0.0019
517	PHE0009475_20462.pep	2OG-FeII_Oxy	223	322	166.9	5.20E-47
518	PHE0007415_17661.pep	EIN3	60	467	781.6	4.80E-232
519	PHE0006614_16129.pep	Cellulase	19	358	-25.9	1.00E-05
520	PHE0010611_22396.pep	FMO-like	25	424	-364.7	5.40E-07
521	PHE0011719_23946.pep	Acyltransferase	69	222	30	8.30E-06
522	PHE0006861_16463.pep	NPH3	166	409	387.6	1.80E-113
523	PHE0006568_16005.pep	DUF26	80	134	56.9	6.70E-14
523	PHE0006568_16005.pep	DUF26	199	253	62.1	1.80E-15

PEP SEQ ID NO	Gene ID	Pfam domain name	Begin	Stop	Score	E-value
523	PHE0006568_16005.pep	Pkinase_Tyr	346	615	114	4.20E-31
523	PHE0006568_16005.pep	Pkinase	346	615	163.2	6.70E-46
524	PHE0010652_22429.pep	FtsJ	21	211	282.4	8.80E-82
525	PHE0008158_18448.pep	PLATZ	22	144	232.3	1.00E-66
526	PHE0003316_20755.pep	Glyco_hydro_2_N	9	180	238.7	1.30E-68
526	PHE0003316_20755.pep	Glyco_hydro_2	182	272	129.9	7.00E-36
526	PHE0003316_20755.pep	Glyco_hydro_2_C	274	593	612.1	5.00E-181
527	PHE0009478_20470.pep	2OG-FeII_Oxy	229	328	171.2	2.50E-48
528	PHE0010395_21760.pep	DUF716	109	266	180.5	4.10E-51
529	PHE0010391_21753.pep	DUF640	28	160	296.6	4.70E-86
530	PHE0010396_21761.pep	DUF716	109	265	175.2	1.60E-49
531	PHE0009511_22422.pep	SAM_decarbox	5	337	710.3	1.30E-210
532	PHE0010614_22399.pep	SOUL	28	211	330.7	2.50E-96
534	PHE0011454_23667.pep	Histone	56	126	103.3	7.20E-28
535	PHE0011443_24001.pep	Histone	61	131	103.3	7.20E-28
536	PHE0011452_23665.pep	Histone	58	132	142.7	9.80E-40
537	PHE0010394_21758.pep	SAP18	29	150	260.5	3.30E-75
540	PHE0010100_21467.pep	Pyr_redox_2	5	285	134.4	3.10E-37
540	PHE0010100_21467.pep	Pyr_redox	147	240	91	3.50E-24
540	PHE0010100_21467.pep	Fer2_BFD	420	472	81.5	2.60E-21
540	PHE0010100_21467.pep	NIR_SIR_ferr	554	621	89.9	7.80E-24
540	PHE0010100_21467.pep	NIR_SIR	629	775	176.5	6.70E-50
541	PHE0011503_23734.pep	GAF	158	307	91.9	1.90E-24
541	PHE0011503_23734.pep	HisKA	343	408	87.5	4.10E-23
541	PHE0011503_23734.pep	HATPase_c	455	586	123.9	4.50E-34
542	PHE0010099_21319.pep	Molybdop_Fe4S4	1	55	91.2	3.30E-24
542	PHE0010099_21319.pep	Molybdopterin	58	475	479.9	3.00E-141
542	PHE0010099_21319.pep	Molydop_binding	570	681	140	6.60E-39
542	PHE0010099_21319.pep	Fer2_BFD	811	863	74.8	2.70E-19
543	PHE0011269_23400.pep	Homeobox	21	78	70.4	5.80E-18
543	PHE0011269_23400.pep	START	215	437	167.3	3.90E-47
546	PHE0011613_23858.pep	AP2	62	136	129.6	8.70E-36
546	PHE0011613_23858.pep	AP2	165	230	109.9	7.50E-30
547	PHE0001582_22064.pep	GAF	195	344	57.2	5.30E-14
547	PHE0001582_22064.pep	HisKA	380	445	22.2	0.00068
547	PHE0001582_22064.pep	Response_reg	645	761	69.4	1.20E-17
548	PHE0007444_22314.pep	Spermine_synth	90	349	414.5	1.50E-121
549	PHE0010543_22298.pep	PBP	19	165	216.7	5.30E-62
550	PHE0010543_22323.pep	PBP	19	157	186	9.30E-53
551	PHE0011081_23033.pep	Sugar_tr	33	465	-7.2	1.20E-06
552	PHE0011075_23026.pep	P-II	73	175	181.5	2.10E-51
553	PHE0010100_21462.pep	Pyr_redox_2	5	285	134.4	3.10E-37
553	PHE0010100_21462.pep	Pyr_redox	147	240	91	3.50E-24
553	PHE0010100_21462.pep	Fer2_BFD	420	472	81.5	2.60E-21
553	PHE0010100_21462.pep	NIR_SIR_ferr	554	621	89.9	7.80E-24
553	PHE0010100_21462.pep	NIR_SIR	629	775	176.5	6.70E-50

PEP SEQ ID NO	Gene ID	Pfam domain name	Begin	Stop	Score	E-value
554	PHE0009640_21775.pep	Auxin_inducible	1	102	151.5	2.10E-42
555	PHE0010092_21307.pep	S1	13	88	51.4	3.00E-12
555	PHE0010092_21307.pep	EIF_2_alpha	125	256	217.3	3.50E-62
556	PHE0004611_24123.pep	PTR2	13	406	283.1	5.40E-82
557	PHE0010093_21308.pep	S1	13	88	50	8.10E-12
557	PHE0010093_21308.pep	EIF_2_alpha	125	256	217.3	3.50E-62
558	PHE0008605_23084.pep	Histone	58	128	105.5	1.60E-28
559	PHE0009939_21100.pep	GDC-P	81	508	1024.4	0
560	PHE0009941_21105.pep	mTERF	2	262	117.5	3.70E-32
561	PHE0009951_21135.pep	Ribosomal_L10e	1	176	487.9	1.20E-143
562	PHE0009943_21127.pep	Ribosomal_L10e	1	176	460.5	2.20E-135
563	PHE0009948_21132.pep	Ribosomal_L10e	1	176	476.4	3.40E-140
564	PHE0011084_23040.pep	Peptidase_S10	54	482	561	1.20E-165
565	PHE0008233_23042.pep	Phi_1	40	314	616.9	1.80E-182
566	PHE0010854_22730.pep	RGS	294	412	23.2	2.80E-06
567	PHE0003797_23051.pep	zf-Dof	34	96	127	5.20E-35
568	PHE0010194_21769.pep	Glyco_hydro_9	52	509	1007.2	5.70E-300
570	PHE0011665_23899.pep	Chloroa_b-bind	63	260	255.3	1.20E-73
571	PHE0012178_24443.pep	Whirly	53	191	263.2	5.30E-76
573	PHE0011064_22994.pep	TPR_1	73	106	33.7	6.50E-07
573	PHE0011064_22994.pep	TPR_2	73	106	33.4	7.80E-07
577	PHE0011446_23659.pep	Histone	58	132	142.9	8.50E-40
578	PHE0001424_22077.pep	Agglutinin	55	193	46.9	6.30E-12
579	PHE0011445_23658.pep	C2	6	87	74.9	2.50E-19
580	PHE0010090_21297.pep	LRRNT_2	33	73	34.2	4.60E-07
580	PHE0010090_21297.pep	LRR_1	78	101	11.5	2
580	PHE0010090_21297.pep	LRR_1	103	127	11.2	2.3
580	PHE0010090_21297.pep	LRR_1	129	151	21.9	0.0024
580	PHE0010090_21297.pep	LRR_1	177	199	16	0.14
580	PHE0010090_21297.pep	LRR_1	226	248	10.3	3.3
580	PHE0010090_21297.pep	LRR_1	274	296	18.9	0.018
580	PHE0010090_21297.pep	LRR_1	298	320	12.1	1.6
580	PHE0010090_21297.pep	LRR_1	322	341	11.2	2.3
580	PHE0010090_21297.pep	LRR_1	395	417	9.8	4.1
580	PHE0010090_21297.pep	LRR_1	444	466	8.2	8.3
580	PHE0010090_21297.pep	LRR_1	468	490	12.8	1.1
580	PHE0010090_21297.pep	LRR_1	492	514	7.7	10
580	PHE0010090_21297.pep	LRR_1	516	538	15.7	0.17
580	PHE0010090_21297.pep	LRR_1	540	562	7.8	10
580	PHE0010090_21297.pep	LRR_1	564	585	8.5	7.4
580	PHE0010090_21297.pep	Pkinase	678	952	107.9	3.00E-29
580	PHE0010090_21297.pep	Pkinase_Tyr	678	952	110.4	5.10E-30
581	PHE0012180_24445.pep	B3	516	619	90.3	6.00E-24
582	PHE0011083_23039.pep	adh_short	44	200	46.7	7.60E-11
583	PHE0011085_23041.pep	Peptidase_S10	31	459	551.6	8.20E-163
584	PHE0012175_24440.pep	CXC	457	498	80.2	6.60E-21

PEP SEQ ID NO	Gene ID	Pfam domain name	Begin	Stop	Score	E-value
584	PHE0012175_24440.pep	CXC	543	584	83.5	6.40E-22
585	PHE0012177_24442.pep	Whirly	91	229	325.2	1.10E-94
587	PHE0010194_21426.pep	Glyco_hydro_9	52	509	1013.8	5.70E-302
588	PHE0011666_23900.pep	Spermine_synth	46	291	503.1	3.20E-148
589	PHE0011448_24160.pep	Histone	25	90	35.4	1.90E-07
590	PHE0008324_18636.pep	DUF1005	256	460	524.8	9.30E-155
591	PHE0006971_16875.pep	Myb_DNA-binding	14	61	44.2	4.40E-10
591	PHE0006971_16875.pep	Myb_DNA-binding	67	112	51.3	3.20E-12
592	PHE0009939_21147.pep	GDC-P	81	508	1024.4	0
593	PHE0009953_21137.pep	Na_sulph_symp	110	578	831.2	5.40E-247
595	PHE0008557_18970.pep	NAD_binding_1	234	350	138.6	1.60E-38
596	PHE0006443_15955.pep	YDG_SRA	360	519	403.5	3.00E-118
596	PHE0006443_15955.pep	Pre-SET	543	639	156.7	6.00E-44
596	PHE0006443_15955.pep	SET	641	771	181.7	1.80E-51

Table 11. pfam module annotation

PEP SEQ ID NO	Gene ID	Pfam domain module	Position
299	PHE0007721_18998.pep	KNOX1::KNOX2::ELK	102-146::153-204::242-263
300	PHE0007756_18009.pep	RPE65	105-591
302	PHE0004988_15925.pep	F-box::LRR_1::LRR_2	4-52::157-179::290-314
303	PHE0007140_21771.pep	MtN3_slv::MtN3_slv	9-98::134-221
304	PHE0006823_16403.pep	Globin	10-149
305	PHE0007440_22555.pep	Ras	14-175
306	PHE0000206_22432.pep	Pkinase::efhand::efhand::efhand	79-337::384-412::456-484::490-518
307	PHE0000598_16824.pep	HLH	385-434
308	PHE0007590_17883.pep	S1	2-70
309	PHE0007588_17881.pep	S1	2-86
310	PHE0007592_17885.pep	S1	1-75
311	PHE0007587_17880.pep	S1	3-75
312	PHE0007624_17923.pep	eIF-1a	5-70
313	PHE0007591_17884.pep	S1	2-68
314	PHE0007623_17922.pep	CN_hydrolase	11-184
315	PHE0007583_17871.pep	MtN3_slv::MtN3_slv	9-98::132-218
316	PHE0006618_16146.pep	S10_plectin	3-98
317	PHE0006791_16374.pep	GUN4	84-227
318	PHE0007620_17918.pep	peroxidase	89-321
319	PHE0006659_16195.pep	MFS_1	10-391
321	PHE0006274_15867.pep	E2F_TDP	130-195

PEP SEQ ID NO	Gene ID	Pfam domain module	Position
322	PHE0006637_16166.pep	PP2C	7-305
323	PHE0006926_16815.pep	VPS28	18-209
324	PHE0006821_16402.pep	PALP	49-358
325	PHE0007687_18057.pep	p450	35-462
326	PHE0007642_17999.pep	PAS_3::PAS_3::Pkinase	211-303::489-581::663-952
327	PHE0007677_18052.pep	FAD_binding_3	5-372
328	PHE0007644_18001.pep	LRRNT_2::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::Pkinase	23-64::97-119::121-143::145-167::169-192::194-216::414-716
329	PHE0007640_17992.pep	CorA	66-448
330	PHE0007678_18053.pep	4HBT	66-151
331	PHE0007649_18006.pep	Ank::Pkinase	108-140::196-455
332	PHE0006726_16298.pep	WD40::WD40	45-83::135-173
333	PHE0006218_8776.pep	Pyr_redox_2::Thioredoxin	85-370::423-528
335	PHE0002554_17876.pep	Chloroa_b-bind	63-261
336	PHE0006807_16388.pep	CCT	352-390
337	PHE0003359_8487.pep	p450	51-489
338	PHE0006542_15765.pep	PMSR	92-245
339	PHE0007622_17921.pep	Spermine_synth	34-279
340	PHE0007630_17956.pep	2OG-FeII_Oxy	190-291
341	PHE0007593_17886.pep	Spermine_synth	28-273
342	PHE0006475_15588.pep	PsbQ	3-162
343	PHE0007619_17915.pep	MBD	11-84
344	PHE0006846_16447.pep	Sterol_desat	1-186
345	PHE0007584_17874.pep	MBD	4-77
346	PHE0003695_17913.pep	LRRNT_2::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::Pkinase	23-64::93-115::117-139::141-163::165-186::189-211::213-235::237-259::260-281::284-306::308-330::332-354::356-378::380-402::404-426::428-450::452-474::476-498::500-521::523-542::648-917
347	PHE0003695_17879.pep	LRRNT_2::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::Pkinase	23-64::93-115::117-139::141-163::165-186::189-211::213-235::237-259::260-281::284-306::308-330::332-354::356-378::380-402::404-426::428-450::452-474::476-498::500-521::523-542::648-917
349	PHE0007645_18002.pep	AP2	115-180
350	PHE0006784_16366.pep	PALP	12-301

PEP SEQ ID NO	Gene ID	Pfam domain module	Position
351	PHE0006171_16491.pep	Glyoxalase	70-214
353	PHE0006906_16796.pep	Proteasome	10-206
354	PHE0006649_16180.pep	AP2	46-110
355	PHE0006809_16390.pep	Prismane	1-548
356	PHE0006918_16808.pep	SWIM	321-353
357	PHE0007813_18219.pep	MtN3_slv::MtN3_slv	9-98::132-218
359	PHE0006825_16405.pep	Cytochrom_C552	27-476
360	PHE0006964_16864.pep	tRNA_anti::tRNA-synt_2	43-122::140-557
361	PHE0008105_18407.pep	CBFD_NFYB_HMF	158-222
362	PHE0006630_16159.pep	Ldh_1_N::Ldh_1_C	90-239::241-413
363	PHE0006966_16868.pep	DNA_photolyase::FAD_binding_7	12-183::214-492
365	PHE0006608_16123.pep	GSHPx	42-150
366	PHE0010462_21459.pep	NUDIX::NUDIX	17-159::190-322
367	PHE0009211_21774.pep	Auxin_inducible	1-106
368	PHE0006256_8775.pep	RPE65	105-591
369	PHE0006989_16918.pep	Pkinase	88-396
370	PHE0007649_18166.pep	Ank::Pkinase	108-140::196-455
371	PHE0006708_16264.pep	DEAD::Helicase_C	57-223::291-367
372	PHE0001117_19094.pep	Homeobox::HALZ	79-133::134-178
373	PHE0006266_8814.pep	Fe_bilin_red	16-244
374	PHE0006795_16378.pep	Na_H_antiport_1	4-380
375	PHE0006956_16853.pep	Molybdop_Fe4S4::Molybdopterin::Molydop_binding::Fer2_BFD	2-56::59-482::578-689::825-877
377	PHE0006824_16404.pep	Globin	14-154
378	PHE0006812_16393.pep	Bac_globin	3-118
379	PHE0006815_16396.pep	Bac_globin	26-146
380	PHE0006806_16387.pep	Bac_globin	26-146
381	PHE0006624_16153.pep	NAD_binding_2::6PGD	3-178::182-473
382	PHE0005006_15823.pep	Chloroa_b-bind	63-242
384	PHE0006683_16229.pep	Response_reg	10-151
385	PHE0006884_16703.pep	Glyco_transf_5::Glycos_transf_1	83-344::384-567
386	PHE0006691_16237.pep	NAD_Gly3P_dh_N::NAD_Gly3P_dh_C	6-165::183-327
387	PHE0006790_16372.pep	Rieske::PaO	220-317::408-507
388	PHE0009143_19932.pep	Histone_HNS	21-129
389	PHE0008406_18830.pep	AA_permease	57-511
390	PHE0008279_18708.pep	Mit_rib_S27	14-93
391	PHE0009142_19931.pep	Histone_HNS	21-130

PEP SEQ ID NO	Gene ID	Pfam domain module	Position
393	PHE0007639_17991.pep	ELFV_dehydrog_N::ELFV_dehydrog	84-214::229-474
394	PHE0007646_18170.pep	F-box::Kelch_1::Kelch_1	36-83::131-185::187-233
395	PHE0007765_18182.pep	GST_N::GST_C	5-79::101-204
396	PHE0008167_18465.pep	Pkinase_Tyr	8-262
398	PHE0008183_18479.pep	LRRNT_2::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::Pkinase_Tyr	23-66::71-93::95-117::119-142::144-166::168-190::192-214::289-311::313-335::337-359::361-384::409-431::457-479::481-503::505-527::529-551::553-575::577-598::695-966
399	PHE0007585_17875.pep	LEA_3	35827
400	PHE0006804_16385.pep	zf-CCCH::zf-CCCH	13-39::149-174
401	PHE0006703_15751.pep	PFK	72-347
402	PHE0007410_17653.pep	Acyltransferase	27-249
403	PHE0006854_16456.pep	YABBY	40-278
404	PHE0007721_21293.pep	KNOX1::KNOX2::ELK	102-146::153-204::242-263
405	PHE0006563_15990.pep	GATase_2::Asn_synthase	2-161::209-450
406	PHE0006619_16147.pep	AA_kinase	15-261
407	PHE0006813_16394.pep	Bac_globin	7-124
408	PHE0006688_16234.pep	Ribosomal_S2	13-229
409	PHE0006635_16164.pep	MGS	28-121
411	PHE0008416_18838.pep	PTR2	115-504
413	PHE0008599_19166.pep	Isoamylase_N::Alpha-amylase	24-115::164-578
414	PHE0008375_18732.pep	Pribosyltran	93-237
416	PHE0008394_18763.pep	Gln-synt_N::Gln-synt_C	68-148::154-406
417	PHE0008395_18764.pep	Gln-synt_N::Gln-synt_C	68-148::154-406
418	PHE0008444_18846.pep	Gln-synt_N::Gln-synt_C	17-97::103-355
419	PHE0006819_16400.pep	Lactamase_B::Flavodoxin_1::Flavin_Reduct	47-236::267-397::424-573
420	PHE0007417_17663.pep	EIN3	60-467
421	PHE0008443_18845.pep	GATase_2::Asn_synthase	2-161::209-450
422	PHE0006818_16399.pep	Lactamase_B::Flavodoxin_1::Rubredoxin	34-227::256-389::423-471
423	PHE0006579_16031.pep	Thi4	63-306
424	PHE0006808_16389.pep	CCT	354-392
425	PHE0008160_18460.pep	TPP_enzyme_N::TPP_enzyme_M::TPP_enzyme_C	45-221::243-376::431-578
426	PHE0007571_17834.pep	GSH_synth_ATP	29-499

PEP SEQ ID NO	Gene ID	Pfam domain module	Position
427	PHE0007586_17877.pep	Chloroa_b-bind	64-261
429	PHE0009729_21717.pep	Homeobox::HALZ	67-123::124-168
430	PHE0006424_15520.pep	PsbW_2	1-133
431	PHE0006392_15480.pep	PsbP	106-322
432	PHE0006589_16093.pep	Thioredoxin	64-167
433	PHE0009258_21487.pep	Na_H_Exchanger	22-441
434	PHE0006680_16226.pep	2OG-FeII_Oxy	187-287
435	PHE0006481_16072.pep	adh_short	33-181
436	PHE0006269_8820.pep	TPR_2::TPR_1::TPR_2::TPR_1::TPR_4::TPR_2::TPR_1	74-107::108-141::142-175::179-212::247-272::281-314::349-382
437	PHE0006783_16365.pep	Aa_trans	32-469
438	PHE0003151_18392.pep	AP2::AP2	318-391::420-485
439	PHE0008272_18723.pep	HSF_DNA-bind	18-188
440	PHE0008277_18706.pep	SNF5	172-400
441	PHE0008109_18411.pep	Sad1_UNC	203-330
442	PHE0008280_18826.pep	Alpha-amylase	14-452
443	PHE0002424_15825.pep	adh_short	43-215
444	PHE0006914_16804.pep	AAA	204-391
445	PHE0006642_16172.pep	p450	42-496
447	PHE0009926_21079.pep	DSPc	24-152
448	PHE0006206_19159.pep	Sina	106-305
449	PHE0008274_18705.pep	BURP	56-280
450	PHE0009510_20526.pep	Spermine_synth	38-283
451	PHE0009937_21097.pep	Spermine_synth	38-283
452	PHE0009927_21080.pep	DSPc	24-162
454	PHE0006445_15956.pep	Allene_ox_cyc	74-253
455	PHE0006987_16893.pep	efhand::efhand	86-114::167-195
456	PHE0004230_15865.pep	E2F_TDP	146-211
457	PHE0006814_16395.pep	Bac_globin	26-144
458	PHE0002773_15881.pep	NTP_transferase	88-366
459	PHE0006060_15842.pep	ubiquitin::ubiquitin::ubiquitin::ubiquitin	5-73::81-149::157-225::233-301
460	PHE0006850_16451.pep	YABBY	6-166
461	PHE0008043_18199.pep	Homeobox::START::MEKHLA	25-85::164-374::693-840
462	PHE0000125_18852.pep	Response_reg::CCT	28-153::457-495
463	PHE0008269_18720.pep	Pkinase	23-279
464	PHE0008264_18712.pep	zf-UBR	40-109
465	PHE0002782_20059.pep	PGI	2-457
466	PHE0009438_20404.pep	GATA	191-226

PEP SEQ ID NO	Gene ID	Pfam domain module	Position
467	PHE0009429_20392.pep	ParBc	70-151
468	PHE0009440_20406.pep	ParBc	11-126
469	PHE0008543_18945.pep	TP_methylase	222-435
470	PHE0006960_16857.pep	Oxidored_molyb::Mo-co_dimer::Cyt-b5::FAD_binding_6::NAD_binding_1	116-301::326-459::515-588::633-740::759-872
471	PHE0009427_20389.pep	IGPD	103-247
472	PHE0006437_15906.pep	Pkinase	39-307
473	PHE0007409_17652.pep	DAGAT	64-340
474	PHE0009426_20387.pep	PHP	2-281
475	PHE0009430_20393.pep	ParBc	44-128
476	PHE0007003_16906.pep	PTPA	96-396
477	PHE0008378_18741.pep	bZIP_1	223-280
478	PHE0008273_18725.pep	zf-CCCH	80-106
479	PHE0008547_18953.pep	Fer2	20-95
480	PHE0006922_16812.pep	Hexokinase_1::Hexokinase_2	44-251::258-501
481	PHE0006920_16810.pep	AAA::Vps4_C	267-458::464-514
482	PHE0008396_18765.pep	Gln-synt_N::Gln-synt_C	68-148::154-406
483	PHE0006957_16854.pep	Gln-synt_N::Gln-synt_C	68-148::154-406
484	PHE0006404_15936.pep	RRM_1::RRM_1	8-77::112-182
485	PHE0006820_16401.pep	Lactamase_B::Flavin_Reduct	47-236::423-572
486	PHE0006690_16236.pep	MFS_1	57-421
487	PHE0006395_15932.pep	LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1	185-206::208-229::231-252::254-272::278-299::349-370::372-394
488	PHE0006759_16384.pep	SHMT	55-453
489	PHE0006576_16028.pep	Myb_DNA-binding::Myb_DNA-binding	11-57::63-108
490	PHE0006979_16885.pep	Tryp_alpha_amyl	33-111
491	PHE0009786_21754.pep	HLH	33-80
492	PHE0006572_16007.pep	Pkinase	11-269
494	PHE0006588_16092.pep	Thioredoxin	56-161
495	PHE0006985_16891.pep	DUF1716	13-118
496	PHE0006875_16688.pep	PSI_PSAK	44-138
497	PHE0006626_16155.pep	Glyco_hydro_17	30-335
498	PHE0006689_16235.pep	Aa_trans	165-548
499	PHE0006799_16382.pep	B12-binding::Radical_SAM	10-135::202-368

PEP SEQ ID NO	Gene ID	Pfam domain module	Position
500	PHE0006959_16856.pep	Oxidored_molyb::Mo-co_dimer::Cyt-b5::FAD_binding_6::NAD_binding_1	143-328::353-486::543-616::660-767::786-899
501	PHE0011615_23861.pep	Pkinase	267-523
502	PHE0006925_16814.pep	Tim17	4-133
503	PHE0006713_16274.pep	APC8::TPR_1::TPR_1::TPR_1	1-161::339-372::373-406::407-440
504	PHE0008447_18848.pep	Lir1	1-126
505	PHE0006674_16221.pep	PTR2	96-505
506	PHE0010613_22398.pep	DAGK_cat	43-184
507	PHE0002720_22558.pep	DnaJ::DnaJ_CXXCXGXG::DnaJ_C	90-151::218-301::314-436
508	PHE0011760_24005.pep	Pkinase	400-656
509	PHE0010612_22397.pep	G-patch	15-59
511	PHE0007411_17654.pep	Acyltransferase	136-283
512	PHE0010617_22402.pep	Isochorismatase	27-210
513	PHE0010610_22395.pep	zf-C3HC4	103-144
514	PHE0006883_16702.pep	Glyco_transf_5::Glycos_transf_1	78-339::379-562
515	PHE0010636_22408.pep	DUF6::TPT	105-230::239-384
516	PHE0011082_23038.pep	adh_short	19-187
517	PHE0009475_20462.pep	2OG-FeII_Oxy	223-322
518	PHE0007415_17661.pep	EIN3	60-467
519	PHE0006614_16129.pep	Cellulase	19-358
520	PHE0010611_22396.pep	FMO-like	25-424
521	PHE0011719_23946.pep	Acyltransferase	69-222
522	PHE0006861_16463.pep	NPH3	166-409
523	PHE0006568_16005.pep	DUF26::DUF26::Pkinase	80-134::199-253::346-615
524	PHE0010652_22429.pep	FtsJ	21-211
525	PHE0008158_18448.pep	PLATZ	22-144
526	PHE0003316_20755.pep	Glyco_hydro_2_N::Glyco_hydro_2::Glyco_hydro_2_C	9-180::182-272::274-593
527	PHE0009478_20470.pep	2OG-FeII_Oxy	229-328
528	PHE0010395_21760.pep	DUF716	109-266
529	PHE0010391_21753.pep	DUF640	28-160
530	PHE0010396_21761.pep	DUF716	109-265
531	PHE0009511_22422.pep	SAM_decarbox	5-337
532	PHE0010614_22399.pep	SOUL	28-211
534	PHE0011454_23667.pep	Histone	56-126
535	PHE0011443_24001.pep	Histone	61-131

PEP SEQ ID NO	Gene ID	Pfam domain module	Position
536	PHE0011452_23665.pep	Histone	58-132
537	PHE0010394_21758.pep	SAP18	29-150
540	PHE0010100_21467.pep	Pyr_redox_2::Fer2_BFD ::NIR_SIR_ferr::NIR_SI R	5-285::420-472::554-621::629-775
541	PHE0011503_23734.pep	GAF::HisKA::HATPase _c	158-307::343-408::455-586
542	PHE0010099_21319.pep	Molybdop_Fe4S4::Moly bdopterin::Molydop_bin ding::Fer2_BFD	1-55::58-475::570-681::811-863
543	PHE0011269_23400.pep	Homeobox::START	21-78::215-437
546	PHE0011613_23858.pep	AP2::AP2	62-136::165-230
547	PHE0001582_22064.pep	GAF::HisKA::Response _reg	195-344::380-445::645-761
548	PHE0007444_22314.pep	Spermine_synth	90-349
549	PHE0010543_22298.pep	PBP	19-165
550	PHE0010543_22323.pep	PBP	19-157
551	PHE0011081_23033.pep	Sugar_tr	33-465
552	PHE0011075_23026.pep	P-II	73-175
553	PHE0010100_21462.pep	Pyr_redox_2::Fer2_BFD ::NIR_SIR_ferr::NIR_SI R	5-285::420-472::554-621::629-775
554	PHE0009640_21775.pep	Auxin_inducible	1-102
555	PHE0010092_21307.pep	S1::EIF_2_alpha	13-88::125-256
556	PHE0004611_24123.pep	PTR2	13-406
557	PHE0010093_21308.pep	S1::EIF_2_alpha	13-88::125-256
558	PHE0008605_23084.pep	Histone	58-128
559	PHE0009939_21100.pep	GDC-P	81-508
560	PHE0009941_21105.pep	mTERF	2-262
561	PHE0009951_21135.pep	Ribosomal_L10e	1-176
562	PHE0009943_21127.pep	Ribosomal_L10e	1-176
563	PHE0009948_21132.pep	Ribosomal_L10e	1-176
564	PHE0011084_23040.pep	Peptidase_S10	54-482
565	PHE0008233_23042.pep	Phi_1	40-314
566	PHE0010854_22730.pep	RGS	294-412
567	PHE0003797_23051.pep	zf-Dof	34-96
568	PHE0010194_21769.pep	Glyco_hydro_9	52-509
570	PHE0011665_23899.pep	Chloroa_b-bind	63-260
571	PHE0012178_24443.pep	Whirly	53-191
573	PHE0011064_22994.pep	TPR_1	73-106
577	PHE0011446_23659.pep	Histone	58-132
578	PHE0001424_22077.pep	Agglutinin	55-193

PEP SEQ ID NO	Gene ID	Pfam domain module	Position
579	PHE0011445_23658.pep	C2	6-87
580	PHE0010090_21297.pep	LRRNT_2::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::Pkinase_Tyr	33-73::78-101::103-127::129-151::177-199::226-248::274-296::298-320::322-341::395-417::444-466::468-490::492-514::516-538::540-562::564-585::678-952
581	PHE0012180_24445.pep	B3	516-619
582	PHE0011083_23039.pep	adh_short	44-200
583	PHE0011085_23041.pep	Peptidase_S10	31-459
584	PHE0012175_24440.pep	CXC::CXC	457-498::543-584
585	PHE0012177_24442.pep	Whirly	91-229
587	PHE0010194_21426.pep	Glyco_hydro_9	52-509
588	PHE0011666_23900.pep	Spermine_synth	46-291
589	PHE0011448_24160.pep	Histone	25-90
590	PHE0008324_18636.pep	DUF1005	256-460
591	PHE0006971_16875.pep	Myb_DNA-binding::Myb_DNA-binding	14-61::67-112
592	PHE0009939_21147.pep	GDC-P	81-508
593	PHE0009953_21137.pep	Na_sulph_symp	110-578
595	PHE0008557_18970.pep	NAD_binding_1	234-350
596	PHE0006443_15955.pep	YDG_SRA::Pre-SET::SET	360-519::543-639::641-771

Table 12. Description of pfam domains

Pfam domain name	Accession number	Gathering cutoff	Domain description
2OG-FeII_Oxy	PF03171.10	11.5	2OG-Fe(II) oxygenase superfamily
4HBT	PF03061.12	20.6	Thioesterase superfamily
6PGD	PF00393.9	-232.3	6-phosphogluconate dehydrogenase, C-terminal domain
AAA	PF00004.19	12.3	ATPase family associated with various cellular activities (AAA)
AA_kinase	PF00696.18	-40	Amino acid kinase family
AA_permease	PF00324.11	-120.8	Amino acid permease
AP2	PF00847.10	0	AP2 domain
APC8	PF04049.4	-19.8	Anaphase promoting complex subunit 8 / Cdc23
Aa_trans	PF01490.8	-128.4	Transmembrane amino acid transporter protein
Acyltransferase	PF01553.11	-0.4	Acyltransferase
Agglutinin	PF07468.2	25	Agglutinin

Pfam domain name	Accession number	Gathering cutoff	Domain description
Allene_ox_cyc	PF06351.2	25	Allene oxide cyclase
Alpha-amylase	PF00128.14	-92.6	Alpha amylase, catalytic domain
Ank	PF00023.20	0	Ankyrin repeat
ApbA	PF02558.6	-16.2	Ketopantoate reductase PanE/ApbA
Asn_synthase	PF00733.11	-52.8	Asparagine synthase
Auxin_inducible	PF02519.5	-15	Auxin responsive protein
B12-binding	PF02310.9	19.6	B12 binding domain
B3	PF02362.12	26.5	B3 DNA binding domain
BURP	PF03181.6	-52	BURP domain
Bac_globin	PF01152.11	-12.7	Bacterial-like globin
C2	PF00168.20	3.7	C2 domain
CBFD_NFYB_HMF	PF00808.13	18.4	Histone-like transcription factor (CBF/NF-Y) and archaeal histone
CCT	PF06203.4	25	CCT motif
CN_hydrolase	PF00795.12	-13.9	Carbon-nitrogen hydrolase
CXC	PF03638.5	25	Tesmin/TSO1-like CXC domain
Cellulase	PF00150.8	-49.3	Cellulase (glycosyl hydrolase family 5)
Chloroa_b-bind	PF00504.12	-31.9	Chlorophyll A-B binding protein
CorA	PF01544.9	-61.3	CorA-like Mg2+ transporter protein
Cyt-b5	PF00173.18	4	Cytochrome b5-like Heme/Steroid binding domain
Cytochrom_C552	PF02335.6	-166	Cytochrome c552
DAGAT	PF03982.3	-151.7	Diacylglycerol acyltransferase
DAGK_cat	PF00781.14	-5.7	Diacylglycerol kinase catalytic domain (presumed)
DAO	PF01266.14	-35	FAD dependent oxidoreductase
DEAD	PF00270.19	7.2	DEAD/DEAH box helicase
DNA_photolyase	PF00875.8	26.1	DNA photolyase
DSPc	PF00782.11	-21.8	Dual specificity phosphatase, catalytic domain
DUF1005	PF06219.3	25	Protein of unknown function (DUF1005)
DUF1716	PF08216.2	25	Eukaryotic domain of unknown function (DUF1716)
DUF26	PF01657.8	0	Domain of unknown function DUF26
DUF6	PF00892.11	20.8	Integral membrane protein DUF6
DUF640	PF04852.3	2.4	Protein of unknown function (DUF640)
DUF716	PF04819.3	-18.4	Family of unknown function (DUF716)
DnaJ	PF00226.21	-8	DnaJ domain
DnaJ_C	PF01556.9	-24	DnaJ C terminal region
DnaJ_CXXCXGXG	PF00684.9	1	DnaJ central domain (4 repeats)
E2F_TDP	PF02319.11	17	E2F/DP family winged-helix DNA-binding domain
EIF_2_alpha	PF07541.2	-4.2	Eukaryotic translation initiation factor 2 alpha subunit
EIN3	PF04873.4	-137.6	Ethylene insensitive 3
ELFV_dehydrog	PF00208.11	-27	Glutamate/Leucine/Phenylalanine/Valine dehydrogenase

Pfam domain name	Accession number	Gathering cutoff	Domain description
ELFV_dehydrog_N	PF02812.8	31.8	Glu/Leu/Phe/Val dehydrogenase, dimerisation domain
ELK	PF03789.4	25	ELK domain
F-box	PF00646.23	13.9	F-box domain
FAD_binding_3	PF01494.9	-136.6	FAD binding domain
FAD_binding_6	PF00970.14	-11.4	Oxidoreductase FAD-binding domain
FAD_binding_7	PF03441.4	25	FAD binding domain of DNA photolyase
FMO-like	PF00743.9	-381.6	Flavin-binding monooxygenase-like
Fe_bilin_red	PF05996.3	25	Ferredoxin-dependent bilin reductase
Fer2	PF00111.17	7	2Fe-2S iron-sulfur cluster binding domain
Fer2_BFD	PF04324.5	25	BFD-like [2Fe-2S] binding domain
Flavin_Reduct	PF01613.8	-18	Flavin reductase like domain
Flavodoxin_1	PF00258.15	6.3	Flavodoxin
FtsJ	PF01728.9	-50.2	FtsJ-like methyltransferase
G-patch	PF01585.13	18.3	G-patch domain
GAF	PF01590.16	23	GAF domain
GATA	PF00320.17	28.5	GATA zinc finger
GATase_2	PF00310.11	-95.1	Glutamine amidotransferases class-II
GDC-P	PF02347.6	-306.2	Glycine cleavage system P-protein
GSHPx	PF00255.10	-16	Glutathione peroxidase
GSH_synth_ATP	PF03917.8	-129.9	Eukaryotic glutathione synthase, ATP binding domain
GSH_synthase	PF03199.6	25	Eukaryotic glutathione synthase
GST_C	PF00043.15	22.3	Glutathione S-transferase, C-terminal domain
GST_N	PF02798.10	14.6	Glutathione S-transferase, N-terminal domain
GUN4	PF05419.3	25	GUN4-like
Gln-synt_C	PF00120.14	-124	Glutamine synthetase, catalytic domain
Gln-synt_N	PF03951.9	9	Glutamine synthetase, beta-Grasp domain
Globin	PF00042.12	-8.8	Globin
Glutaredoxin	PF00462.14	17.2	Glutaredoxin
Glyco_hydro_17	PF00332.9	-152.3	Glycosyl hydrolases family 17
Glyco_hydro_2	PF00703.11	-19.3	Glycosyl hydrolases family 2, immunoglobulin-like beta-sandwich domain
Glyco_hydro_2_C	PF02836.7	-127.7	Glycosyl hydrolases family 2, TIM barrel domain
Glyco_hydro_2_N	PF02837.8	-46.9	Glycosyl hydrolases family 2, sugar binding domain
Glyco_hydro_9	PF00759.9	-246.7	Glycosyl hydrolase family 9
Glyco_transf_5	PF08323.2	-114.9	Starch synthase catalytic domain
Glycos_transf_1	PF00534.10	-7.3	Glycosyl transferases group 1
Glyoxalase	PF00903.15	12.1	Glyoxalase/Bleomycin resistance protein/Dioxygenase superfamily
HALZ	PF02183.8	17	Homeobox associated leucine zipper

Pfam domain name	Accession number	Gathering cutoff	Domain description
HATPase_c	PF02518.16	22.4	Histidine kinase-, DNA gyrase B-, and HSP90-like ATPase
HLH	PF00010.16	8.3	Helix-loop-helix DNA-binding domain
HSF_DNA-bind	PF00447.8	-70	HSF-type DNA-binding
Helicase_C	PF00271.21	2.1	Helicase conserved C-terminal domain
Hexokinase_1	PF00349.11	-110.3	Hexokinase
Hexokinase_2	PF03727.6	-131.3	Hexokinase
HisKA	PF00512.15	10.3	His Kinase A (phosphoacceptor) domain
Histone	PF00125.14	17.4	Core histone H2A/H2B/H3/H4
Histone_HNS	PF00816.11	25	H-NS histone family
Homeobox	PF00046.19	-4.1	Homeobox domain
IGPD	PF00475.9	25	Imidazoleglycerol-phosphate dehydratase
Isoamylase_N	PF02922.8	-6.5	Isoamylase N-terminal domain
Isochorismatase	PF00857.11	-46	Isochorismatase family
KNOX1	PF03790.4	25	KNOX1 domain
KNOX2	PF03791.4	25	KNOX2 domain
KR	PF08659.1	-74.3	KR domain
Kelch_1	PF01344.15	7.8	Kelch motif
Kelch_2	PF07646.5	14	Kelch motif
LEA_3	PF03242.4	25	Late embryogenesis abundant protein
LRRNT_2	PF08263.3	18.6	Leucine rich repeat N-terminal domain
LRR_1	PF00560.23	7.7	Leucine Rich Repeat
LRR_2	PF07723.3	6	Leucine Rich Repeat
Lactamase_B	PF00753.17	24.6	Metallo-beta-lactamase superfamily
Ldh_1_C	PF02866.8	-13	lactate/malate dehydrogenase, alpha/beta C-terminal domain
Ldh_1_N	PF00056.13	-31.3	lactate/malate dehydrogenase, NAD binding domain
Lir1	PF07207.2	25	Light regulated protein Lir1
MBD	PF01429.10	12.9	Methyl-CpG binding domain
MEKHLA	PF08670.1	-59.7	MEKHLA domain
MFS_1	PF07690.6	23.5	Major Facilitator Superfamily
MGS	PF02142.12	3	MGS-like domain
Miro	PF08477.3	10.8	Miro-like protein
Mit_rib_S27	PF08293.2	25	Mitochondrial ribosomal subunit S27
Mo-co_dimer	PF03404.6	25	Mo-co oxidoreductase dimerisation domain
Molybdop_Fe4S4	PF04879.6	13.6	Molybdopterin oxidoreductase Fe4S4 domain
Molybdopterin	PF00384.12	-50	Molybdopterin oxidoreductase
Molydop_binding	PF01568.11	1.1	Molydopterin dinucleotide binding domain
MtN3_slv	PF03083.6	9.7	MtN3/saliva family
Myb_DNA-binding	PF00249.21	14	Myb-like DNA-binding domain
NAD_Gly3P_dh_C	PF07479.4	-50.8	NAD-dependent glycerol-3-phosphate dehydrogenase C-terminus
NAD_Gly3P_dh_N	PF01210.13	-44	NAD-dependent glycerol-3-phosphate

Pfam domain name	Accession number	Gathering cutoff	Domain description
			dehydrogenase N-terminus
NAD_binding_1	PF00175.11	-3.9	Oxidoreductase NAD-binding domain
NAD_binding_2	PF03446.5	-63.5	NAD binding domain of 6-phosphogluconate dehydrogenase
NIR_SIR	PF01077.12	-19.6	Nitrite and sulphite reductase 4Fe-4S domain
NIR_SIR_ferr	PF03460.7	2.4	Nitrite/Sulfite reductase ferredoxin-like half domain
NPH3	PF03000.5	25	NPH3 family
NTP_transferase	PF00483.13	-90.5	Nucleotidyl transferase
NUDIX	PF00293.18	0.2	NUDIX domain
Na_H_Exchanger	PF00999.11	-67.9	Sodium/hydrogen exchanger family
Na_H_antiport_1	PF06965.2	-270.8	Na+/H+ antiporter 1
Na_sulph_symp	PF00939.9	-259	Sodium:sulfate symporter transmembrane region
Oxidored_molyb	PF00174.9	-39.6	Oxidoreductase molybdopterin binding domain
P-II	PF00543.12	-29	Nitrogen regulatory protein P-II
PALP	PF00291.15	-70	Pyridoxal-phosphate dependent enzyme
PAS	PF00989.14	0	PAS fold
PAS_3	PF08447.1	13.4	PAS fold
PBP	PF01161.10	-20.6	Phosphatidylethanolamine-binding protein
PFK	PF00365.10	-132	Phosphofructokinase
PGI	PF00342.9	-168.9	Phosphoglucose isomerase
PHP	PF02811.9	13.8	PHP domain
PLATZ	PF04640.4	20	PLATZ transcription factor
PMSR	PF01625.11	-62	Peptide methionine sulfoxide reductase
PP2C	PF00481.12	-44	Protein phosphatase 2C
PSI_PSAK	PF01241.9	25	Photosystem I psaG / psaK
PTPA	PF03095.5	-106	Phosphotyrosyl phosphate activator (PTPA) protein
PTR2	PF00854.12	-50	POT family
PaO	PF08417.2	25	Pheophorbide a oxygenase
ParBc	PF02195.8	25	ParB-like nuclease domain
Peptidase_S10	PF00450.12	-198	Serine carboxypeptidase
Phi_1	PF04674.3	25	Phosphate-induced protein 1 conserved region
Pkinase	PF00069.15	-70.3	Protein kinase domain
Pkinase_Tyr	PF07714.7	65	Protein tyrosine kinase
Pre-SET	PF05033.6	3.9	Pre-SET motif
Pribosyltran	PF00156.17	2	Phosphoribosyl transferase domain
Prismane	PF03063.10	-169.3	Prismane/CO dehydrogenase family
Proteasome	PF00227.16	-36.7	Proteasome A-type and B-type
PsbP	PF01789.7	25	PsbP
PsbQ	PF05757.2	25	Oxygen evolving enhancer protein 3 (PsbQ)

Pfam domain name	Accession number	Gathering cutoff	Domain description
PsbW_2	PF07123.2	-25.8	Photosystem II reaction centre W protein (PsbW)
Pyr_redox	PF00070.17	5	Pyridine nucleotide-disulphide oxidoreductase
Pyr_redox_2	PF07992.4	-20	Pyridine nucleotide-disulphide oxidoreductase
RGS	PF00615.9	10	Regulator of G protein signaling domain
RPE65	PF03055.6	-156.5	Retinal pigment epithelial membrane protein
RRM_1	PF00076.12	17.7	RNA recognition motif. (a.k.a. RRM, RBD, or RNP domain)
Radical_SAM	PF04055.11	8.5	Radical SAM superfamily
Ras	PF00071.12	-69.9	Ras family
Response_reg	PF00072.14	4	Response regulator receiver domain
Ribosomal_L10e	PF00826.8	25	Ribosomal L10
Ribosomal_S2	PF00318.10	-22	Ribosomal protein S2
Rieske	PF00355.16	-7	Rieske [2Fe-2S] domain
Rubredoxin	PF00301.10	12.1	Rubredoxin
S1	PF00575.13	16.8	S1 RNA binding domain
S10_plectin	PF03501.5	25	Plectin/S10 domain
SAM_decarbox	PF01536.7	-154	Adenosylmethionine decarboxylase
SAP18	PF06487.3	-49.7	Sin3 associated polypeptide p18 (SAP18)
SET	PF00856.18	23.5	SET domain
SHMT	PF00464.10	-238.5	Serine hydroxymethyltransferase
SNF5	PF04855.3	25	SNF5 / SMARCB1 / INI1
SOUL	PF04832.3	25	SOUL heme-binding protein
START	PF01852.10	-20.7	START domain
SWIM	PF04434.8	11	SWIM zinc finger
Sad1_UNC	PF07738.3	-20.4	Sad1 / UNC-like C-terminal
Sina	PF03145.7	-48.4	Seven in absentia protein family
Spermine_synth	PF01564.7	-93.8	Spermine/spermidine synthase
Sterol_desat	PF01598.8	-13	Sterol desaturase
Sugar_tr	PF00083.14	-85	Sugar (and other) transporter
TPP_enzyme_C	PF02775.11	19.7	Thiamine pyrophosphate enzyme, C-terminal TPP binding domain
TPP_enzyme_M	PF00205.12	-8.1	Thiamine pyrophosphate enzyme, central domain
TPP_enzyme_N	PF02776.8	-70	Thiamine pyrophosphate enzyme, N-terminal TPP binding domain
TPR_1	PF00515.18	7.7	Tetratricopeptide repeat
TPR_2	PF07719.7	20.1	Tetratricopeptide repeat
TPR_4	PF07721.5	15	Tetratricopeptide repeat
TPT	PF03151.7	-15.3	Triose-phosphate Transporter family
TP_methylase	PF00590.10	-38	Tetrapyrrole (Corrin/Porphyrin) Methylases
Thi4	PF01946.7	-65.1	Thi4 family
Thioredoxin	PF00085.10	-25.7	Thioredoxin

Pfam domain name	Accession number	Gathering cutoff	Domain description
Tim17	PF02466.9	2.7	Tim17/Tim22/Tim23 family
Tryp_alpha_amyl	PF00234.12	-4	Protease inhibitor/seed storage/LTP family
UAA	PF08449.2	-146.2	UAA transporter family
VPS28	PF03997.3	25	VPS28 protein
Vps4_C	PF09336.1	-4.6	Vps4 C terminal oligomerisation domain
WD40	PF00400.22	21.5	WD domain, G-beta repeat
Whirly	PF08536.2	25	Whirly transcription factor
YABBY	PF04690.4	25	YABBY protein
YDG_SRA	PF02182.8	25	YDG/SRA domain
adh_short	PF00106.15	-40.2	short chain dehydrogenase
bZIP_1	PF00170.11	24.5	bZIP transcription factor
bZIP_2	PF07716.5	15	Basic region leucine zipper
eIF-1a	PF01176.9	20	Translation initiation factor 1A / IF-1
efhand	PF00036.22	21.7	EF hand
mTERF	PF02536.5	-60	mTERF
p450	PF00067.12	-105	Cytochrome P450
peroxidase	PF00141.13	-10	Peroxidase
tRNA-synt_2	PF00152.11	-168.2	tRNA synthetases class II (D, K and N)
tRNA_anti	PF01336.15	8	OB-fold nucleic acid binding domain
ubiquitin	PF00240.13	19.4	Ubiquitin family
zf-C3HC4	PF00097.15	16	Zinc finger, C3HC4 type (RING finger)
zf-CCCH	PF00642.15	0	Zinc finger C-x8-C-x5-C-x3-H type (and similar)
zf-Dof	PF02701.6	25	Dof domain, zinc finger
zf-UBR	PF02207.10	25	Putative zinc finger in N-recognin (UBR box)

Example 9. Selection of transgenic plants with enhanced agronomic trait(s)

This example illustrates the preparation and identification by selection of transgenic seeds and plants derived from transgenic plant cells of this invention where the plants and seed are identified by screening for a transgenic plant having an enhanced agronomic trait imparted by expression of a protein selected from the group including the homologous proteins identified in Example 6. Transgenic plant cells of corn, soybean, cotton, canola, alfalfa, wheat and rice are transformed with recombinant DNA for expressing each of the homologs identified in Example 6. Plants are regenerated from the transformed plant cells and used to produce progeny plants and seed that are screened for enhanced water use efficiency, enhanced cold tolerance, increased yield, enhanced nitrogen use efficiency, enhanced seed protein and enhanced seed oil. Plants are identified exhibiting enhanced traits imparted by expression of the homologous proteins.

What is claimed is:

1. A plant cell nucleus with stably integrated, recombinant DNA construct, wherein said recombinant DNA construct comprises a promoter that is functional in a plant cell and that is operably linked to a DNA segment encoding a protein comprising an amino acid sequence of SEQ ID NO: 309; and

wherein said recombinant DNA construct is stably integrated into a chromosome in said plant cell nucleus which is selected by screening a population of transgenic plants that have said recombinant DNA construct and an enhanced trait as compared to control plants that do not have said recombinant DNA construct in their nuclei; and

wherein said enhanced trait is selected from group of enhanced traits consisting of enhanced water use efficiency, enhanced cold tolerance, enhanced heat tolerance, enhanced high salinity tolerance, enhanced shade tolerance, increased yield, enhanced nitrogen use efficiency, enhanced seed protein and enhanced seed oil.

2. A recombinant DNA construct comprising a promoter that is functional in a plant cell and that is operably linked to a DNA segment that encodes:

a. at least one protein having an amino acid sequence comprising a Pfam domain module selected from the group consisting of zf-CCCH, PALP, GAF::HisKA::HATPase_c, TPR_2::TPR_1::TPR_2::TPR_1::TPR_4::TPR_2::TPR_1, efhand::efhand, Spermine_synth, ELFV_dehydrog_N::ELFV_dehydrog, PFK, PAS_3::PAS_3::Pkinase, S1, GDC-P, SWIM, B12-binding::Radical_SAM, S1, LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1, Chloroa_b-bind, LRRNT_2::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::Pkinase, WD40::WD40, YABBY, Ldh_1_N::Ldh_1_C, Sina, Na_H_antiport_1, ParBc, YABBY, Histone, Fe_bilin_red, Tryp_alpha_amyl, Pyr_redox_2::Thioredoxin, E2F_TDP, CN_hydrolase, YDG_SRA::Pre-SET::SET, APC8::TPR_1::TPR_1::TPR_1, Ras, tRNA_anti::tRNA-synt_2, Auxin_inducible, PGI, S1, Chloroa_b-bind, Bac_globin, Glyco_hydro_17, MGS, Spermine_synth, LRRNT_2::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::Pkinase, Aa_trans, LRRNT_2::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR

_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::Pkinase_Tyr, Gln-synt_N::Gln-synt_C, SAP18, TPP_enzyme_N::TPP_enzyme_M::TPP_enzyme_C, zf-C3HC4, eIF-1a, RPE65, PBP, Pkinase, AA_permease, F-box::LRR_1::LRR_2, zf-CCCH::zf-CCCH, Lactamase_B::Flavodoxin_1::Flavin_Reduct, Bac_globin, DSPc, adh_short, Tim17, Oxidored_molyb::Mo-co_dimer::Cyt-b5::FAD_binding_6::NAD_binding_1, ubiquitin::ubiquitin::ubiquitin::ubiquitin, MBD, CXC::CXC, HSF_DNA-bind, Spermine_synth, AP2, Peptidase_S10, PALP, EIN3, Gln-synt_N::Gln-synt_C, 2OG-FeII_Oxy, Glyco_hydro_9, GDC-P, B3, PTPA, Acyltransferase, Isochorismatase, FMO-like, Molybdop_Fe4S4::Molybdopterin::Molydop_binding::Fer2_BFD, Lir1, Prismane, Fer2, DEAD::Helicase_C, Molybdop_Fe4S4::Molybdopterin::Molydop_binding::Fer2_BFD, KNOX1::KNOX2::ELK, Glyoxalase, Sad1_UNC, Bac_globin, VPS28, PP2C, Pkinase::efhand::efhand::efhand, LEA_3, Peptidase_S10, Pkinase, CBFD_NFYB_HMF, Gln-synt_N::Gln-synt_C, Pyr_redox_2::Fer2_BFD::NIR_SIR_ferr::NIR_SIR, NUDIX::NUDIX, FAD_binding_3, GST_N::GST_C, SAM_decarbox, Acyltransferase, NTP_transferase, G-patch, 2OG-FeII_Oxy, Gln-synt_N::Gln-synt_C, AAA::Vps4_C, Histone, Pkinase, TPR_1, F-box::Kelch_1::Kelch_1, Spermine_synth, Bac_globin, Bac_globin, zf-UBR, Homeobox::HALZ, Whirly, NAD_binding_1, PTR2, EIN3, 4HBT, adh_short, 2OG-FeII_Oxy, P-II, Myb_DNA-binding::Myb_DNA-binding, DAGK_cat, AP2, MFS_1, Chloroa_b-bind, DUF716, zf-Dof, CCT, Homeobox::HALZ, Histone, 2OG-FeII_Oxy, Globin, Pyr_redox_2::Fer2_BFD::NIR_SIR_ferr::NIR_SIR, Whirly, PsbP, bZIP_1, LRRNT_2::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::Pkinase_Tyr, Phi_1, BURP, Sterol_desat, DSPc, SNF5, Acyltransferase, GATase_2::Asn_synthase, adh_short, Homeobox::START, Pkinase, ParBc, SOUL, DNA_photolyase::FAD_binding_7, Pkinase_Tyr, NAD_Gly3P_dh_N::NAD_Gly3P_dh_C, Na_H_Exchanger, peroxidase, Oxidored_molyb::Mo-co_dimer::Cyt-b5::FAD_binding_6::NAD_binding_1, Hexokinase_1::Hexokinase_2, DUF716, S10_plectin, Thi4, p450, CCT, adh_short, PSI_PSAK, DUF640, Thioredoxin, Globin, Ank::Pkinase, DAGAT, RPE65, Ank::Pkinase, GSHPx, Gln-synt_N::Gln-synt_C, MtN3_slv::MtN3_slv, Allene_ox_cyc, IGPD, MBD, CorA, Response_reg, Histone, AAA, Ribosomal_L10e, Pkinase, DUF26::DUF26::Pkinase, p450, mTERF, AA_kinase, PBP, GUN4, Lactamase_B::Flavodoxin_1::Rubredoxin, C2, RRM_1::RRM_1, Histone, Alpha-amylase,

HLH, Thioredoxin, Histone_HNS, Myb_DNA-binding::Myb_DNA-binding, Cytochrom_C552, AP2::AP2, MtN3_slv::MtN3_slv, SHMT, ParBc, Mit_rib_S27, Ribosomal_S2, KNOX1::KNOX2::ELK, MFS_1, Glyco_transf_5::Glycos_transf_1, Cellulase, Ribosomal_L10e, Spermine_synth, Glyco_hydro_2_N::Glyco_hydro_2::Glyco_hydro_2_C, TP_methylase, AP2::AP2, Histone, Response_reg::CCT, Histone_HNS, DUF1716, p450, GATA, Pkinase, Sugar_tr, Aa_trans, Pribosyltran, Ribosomal_L10e, HLH, PMSR, DnaJ::DnaJ_CXXCXGXG::DnaJ_C, DUF1005, Glyco_transf_5::Glycos_transf_1, Spermine_synth, S1::EIF_2_alpha, RGS, Na_sulph_symp, S1, MtN3_slv::MtN3_slv, Lactamase_B::Flavin_Reduct, LRRNT_2::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::Pkinase, Chloroa_b-bind, PTR2, Agglutinin, PLATZ, NPH3, Auxin_inducible, PTR2, GAF::HisKA::Response_reg, PsbQ, GSH_synth_ATP, GATase_2::Asn_synthase, PHP, FtsJ, DUF6::TPT, Proteasome, PsbW_2, Glyco_hydro_9, NAD_binding_2::6PGD, S1::EIF_2_alpha, Homeobox::START::MEKHLA, S1, Isoamylase_N::Alpha-amylase, E2F_TDP, and Rieske::PaO;

b. a protein comprising an amino acid sequence with at least 90% identity to a consensus amino acid sequence selected from the group consisting of SEQ ID NO: 30526 through 30550;

c. a protein comprising an amino acid sequence selected from the group consisting of SEQ ID NOs 352, 493, 533 and 544, and homologs thereof listed in Table 8;

d. a protein having an amino acid sequence having at least 70% identity to an amino acid sequence selected from the group consisting of SEQ ID NOs 320 and 383; or

e. a protein having an amino acid sequence selected from the group consisting of SEQ ID NO: 299 through SEQ ID NO: 30468;

and wherein said recombinant DNA construct is stably integrated into a chromosome in a plant cell nucleus which is selected by screening a population of transgenic plants that have said recombinant DNA construct and an enhanced trait as compared to control plants that do not have said recombinant DNA construct in their nuclei; and wherein said enhanced trait is selected from group of enhanced traits consisting of enhanced water use efficiency, enhanced cold tolerance, enhanced heat tolerance, enhanced high salinity tolerance, enhanced shade

tolerance, increased yield, enhanced nitrogen use efficiency, enhanced seed protein and enhanced seed oil.

3. A transgenic plant cell nucleus comprising a recombinant DNA construct of claim 2.

4. A transgenic plant cell having a plant cell nucleus of claim 3.

5. The transgenic plant cell of claim 4 wherein said transgenic plant cell is homozygous for said recombinant DNA construct.

6. The transgenic plant cell of claim 4 further comprising DNA expressing a protein that provides tolerance from exposure to an herbicide applied at levels that are lethal to a wild type of said plant cell.

7. The transgenic plant cell of claim 5 wherein said herbicide is a glyphosate, dicamba, or glufosinate compound.

8. A transgenic plant comprising a plurality of plant cells of claim 4.

9. The transgenic plant of claim 8 wherein said transgenic plant is homozygous for said recombinant DNA construct.

10. A transgenic seed comprising a recombinant DNA construct of claim 2.

11. The transgenic seed of claim 10 from a corn, soybean, cotton, canola, alfalfa, wheat or rice plant.

12. A transgenic pollen grain comprising a recombinant DNA construct of claim 2.

13. A method for manufacturing transgenic seeds that can be used to produce a crop of transgenic plants with an enhanced trait resulting from expression of a DNA segment in a plant

cell nucleus comprising a recombinant DNA construct of claim 2, wherein said method comprises:

(a) screening a population of plants for said enhanced trait and said recombinant DNA construct, wherein individual plants in said population can exhibit said trait at a level less than, essentially the same as or greater than the level that said trait is exhibited in control plants which do not contain said recombinant DNA construct, wherein said enhanced trait is selected from the group of enhanced traits consisting of enhanced water use efficiency, enhanced cold tolerance, enhanced heat tolerance, enhanced high salinity tolerance, enhanced shade tolerance, increased yield, enhanced nitrogen use efficiency, enhanced seed protein and enhanced seed oil;

(b) selecting from said population one or more plants that exhibit said trait at a level greater than the level that said trait is exhibited in control plants; and

(c) collecting seeds from selected plant selected from step b.

14. The method of claim 13, wherein said method further comprises:

(a) verifying that said recombinant DNA construct is stably integrated in said selected plants; and

(b) analyzing tissue of said selected plant to determine the expression of a protein having the function of a protein having an amino acid sequence selected from the group consisting of one of SEQ ID NO: 299 through SEQ ID NO: 30468.

15. The method of claim 14 wherein said seed is corn, soybean, cotton, alfalfa, canola wheat or rice seed.

16. A method of producing hybrid corn seed comprising:

(a) acquiring hybrid corn seed from a herbicide tolerant corn plant which also has a stably-integrated, recombinant DNA construct of claim 2;

(b) producing corn plants from said hybrid corn seed, wherein a fraction of the plants produced from said hybrid corn seed is homozygous for said recombinant DNA construct, a fraction of the plants produced from said hybrid corn seed is hemizygous for said recombinant DNA construct, and a fraction of the plants produced from said hybrid corn seed has none of said recombinant DNA construct;

(c) selecting corn plants which are homozygous or hemizygous for said recombinant DNA construct by treating with an herbicide;

(d) collecting seeds from herbicide-treated-surviving corn plants and planting said seed to produce further progeny corn plants;

(e) repeating steps (c) and (d) at least once to produce an inbred corn line; and

(f) crossing said inbred corn line with a second corn line to produce hybrid corn seed.

Fig. 1

```
SEQ ID NO: homolog SEQ ID NOs
4210       ------------------------------------------------------------
4213       ------------------------------------------------------------
23796      ----------------------MASNIMQRCNVFMSERVKLHAAMLALQFGYAGFHIVS
5323       ----------------------MSP---------VPERTKLHIAMAVFQTGYAGNHVIM
9197       ------------------------MVHGRLCNRDGWILTAMVVTEFSNVGVNTLV
22010      ------------------------MVHGRLCNRDGWILTAMVVTEFSNVGVNTLV
2187       ------------------------------------------------------------
26922      --------------------------MAGAVSLWRREAVFLTAMLAGETSIVGLSTLF
12208      --------------------------MAGAVSLWRREAVFLTAMLAGETSIVGLSTLF
27336      --------------------------MAGAVSLWRREAVFLTAMLAGETSIVGLSTLF
19871      ----------------------MPIMAGTASPWRREAVFLTAMLVVETSVVGISTLF
11517      ------------------------------------------------------------
8202       ----------------------MPIMAGTASPWRREAVFLTAMLVVETSVVGISTLF
6010       ------------------------------------------------------------
16802      --------------------------MARTVSLWRREAVFLTAMLATETGVVGISTLF
301        ------------------------------------------------------------
2040       ------------------------------MTRKYFQREVLPVTALVIMECANVGLNTLF
6920       ------------------------------------------------------------
5087       --------------------MKKEMI---------EEVVIVGGLVMVQFVYAGNSLLM
5965       MEGLLSYRSNVLRNIMREREKDRKKMLISEIFSENEDEEVVIIGGLVMVQFVYAGNSLLM
3372       --------------------------MAAVAMADGGGKKPYVVAVAIQAINTGTFVVS
consensus  --------------------------xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
30526

------------------------------------------------------------
------------------------------------------------------------
RAALNMGVSKVVFPVYRNILALMLIGPCAYFLEKKERPALTLSFLIQFFLLALCGITGQS
RFALNLGVSKLIFPLYRNIIALSVLAPSAFFLEKKERPRMTTSLLIQFFLLGLVGITLNQ
KAATSKGLSPFVVLVYSYTFGSLLLLPLTFFSFRSRSLPPLTFSILCNMGILGLIASAFQ
KAATSKGLSPFVVLVYSYTFGSLLLLPLTFFSFR----------------------SAFQ
------------------------------------------------------------MYV
KVATSKGLNIYPFLSYSYLLASLLLLPSLFFTN--------SASILSKIGLLGFLGSMYV
KVATSKGLNIYPFLSYSYLLASLLLLPSLFFTNRSRSLPPLSASILSKIGLLGFLGSMYV
KVATSKGLNIYPFLSYSYLLASLLLLPSLFFTNRSRSLPPLSASILSKIGLLGFLGSMYV
KFATSKGLNIYPFLGYSYLLASLLLLPSLFFTNRSSSLPPLSVSILSKIGLLGFLGSMYV
------------------------------------------------------------MYV
KFATSKGLNIYPFLGYSYLLASLLLLPSLFFTNRSSSLPPLSVSILSKIGLLGFLGSMYV
------------------------------------------------------------MYV
KVATSKGLNLYAFLGYSYLLASLLLLPSLFFTDRSRSLPPLSLSILSKIGLLGLLGSMYV
------------------------------------------------------------
KAATLQGMGFHVFIVYSYGLAALLLLPSLFFSYRSRTLPPMNFSILYKVVLLGLIGCCSN
------------------------------------------------------------
SYLMSLGLGPFTIVIFSTFATFLILSPFAILFERKQWPKELSARLIGKLVLISFAGVTLF
SYLMSLSLGPLTIVIFSTFATFLILSPFAILFERKQWPDELSPRLIGKLVLISFAGVTLF
KAAFDSGMYTYIFVFYRLAAATALLVPIALINCACRRSQSTTSTPAQAMSCRLLFKLFLC
xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx

------------------------------------------------------------
------------------------------------------------------------
RIL-----------------------------------------SLR-IVLHIPTFA
GFY-----------------------------------------IFG-LDNTSPTFA
ILG-----------------------------------------YNG-IKYSSPTLS
ILG-----------------------------------------YNG-IKYSSPTLS
```

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Transgenic Plants With Enhanced Agronomic Traits
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```
ITG-------------------------------------------GIG-IEYSNPTLA
ITG-------------------------------------------GIG-IEYSNPTLA
ITG-------------------------------------------GIG-IEYSNPTLA
ITG-------------------------------------------GIG-IEYSNPTLA
ITG-------------------------------------------YIG-IEYSSPTLA
ITG-------------------------------------------YIG-IEYSSPTLA
ITG-------------------------------------------YIG-IEYSSPTLA
ITG-------------------------------------------YIG-IEYSSPTLA
ITG-------------------------------------------YIG-IEYSSPTLA
------------------------------------------------------------
IMG-------------------------------------------YTG-INYSSPTLA
------------------------------------------------------------
QSL-------------------------------------------FLEGIRLTSPAMA
QTL-------------------------------------------FLEGIRLTSPAMA
AFLGSAGLTLQALTKFVKKSCQEVGGFTSLLSSSLLQGKAEHLHPQVYHASLKQTSATVA
xxx-------------------------------------------xxx-ixxxxptxa


------------------------------------------------------------
------------------------------------------------------------
SAIQNSVPAITFIMAAALRLEKVHISRRDGLAKIIGTVACVSGATIITLYKGPPITHIWR
SATENAVPAVSFLMAALLGIEKVELKRRDGVAKVVGTFVSVAGSLAITLYKGP---TIYQ
SAMSNVNPAFTFILAVVFRMENISLGKKSSVAKVLGTILSIIGALVVTLYHGP-------
SAMSNVNPAFTFILAVVFRMENISLGKKSSVAKVLGTILSIIGALVVTLYHGP-------
SAIGNIVPALTFILAVIFRMEKVSFKERSSVAKVMGTILSLIGAFVVIFYHGPRVFVASS
SAIGNIVPALTFILAVIFRMEKVSFKERSSVAKVMGTILSLIGAFVVIFYHGPRVFVASS
SAIGNIVPALTFILAVIFRMEKVSFKERSSVAKVMGTILSLIGAFVVIFYHGPRVFVASS
SAIGNIVPALTFILAVIFRMEKVSFKERSSVAKVMGTILSLIGAFVVIFYHGPRVFVASS
SAINNITPALTFILAIIFRMEKVSFKERSSLAKLMGTILSLIGALVVIFYHGPRVFLASS
SAINNITPALTFILAIIFRMEKVSFKERSSLAKLMGTILSLIGALVVIFYHGPRVFLASS
SAINNITPALTFILAIIFRMEKVSFKERSSLAKLMGTILSLIGALVVIFYHGPRVFLASS
SAINNITPALTFILAIIFRMEKVSFKERSSLAKLMGTILSLIGALVVIFYHGPRVFLASS
SAISNITPALTFILAIIFRMEKVSFKERSSVAKVMGTILSLIGALVVVLYHGPRVFVASS
-------------------MEKASFKEKSSVAKMVGTIVSLVGALVVVLYHGPRVFTPSS
SAISNLTPAFTFLLAILFRMESVSFKRTSSVAKMLGTIVSIGGAFIVTLYNGPVVINMSP
-------------------MERIYWRHFSSQAKAIGTIVSMAGAFVVILYKGPPILKIHS
TAMPNLAPGLIFFIAWIVRLEKMNMKCVYSKLKILGTLLCVFGALTMSLMHSASIIQDEK
TAMPNLAPGLIFFIAWMVRLEKMDMKCVYSKLKILGTLLCVFGALTMSLMHSASIIQDEK
SAATNSMPVVTFLLALVLRMETIKLRRRSGLGKLAGVALCLAGVLVIAFYVGPSIRPLAH
saxxnxxpxxxfxxaxxxrmexxxxxxxssxakxxgtxxsxxgaxxxxxyxgpxxxxxxx


---------FLPDSKHVTASEDDNWILGCLLLLASSVFWSCWMIMQVPISSSCPDHVLST
---------FLPDSKHVTASEDDNWILGCLLLLASSVFWSCWMIMQVPISSSCPDHVLST
PNLEVTASYFKAFQGNDLSAKSENWTLGCIYLLGNCLAWSGWIVLQAPVLKRYPARLSVT
PSLRSMD---QPTNAGEAEEQNKNWTLGCVCLMGHCLCWSSWIVLQSPLLKRYPARYSFV
----------------MLMSSHSDWIIGGGLLALQYILVSVSYLVMAHTMGRYPSAVVVT
----------------MLMSSHSDWIIGGGLLALQYILVSVSYLVMAHTMGRYPSAVVVT
P-----PYLNFRQLSPPLSSSKSDWLIGGAILTIQGIFVSVSFILQTHIMREYPEAFTVS
P-----PYLNFRQLSPPLSSSKSDWLIGGAILTIQGIFVSVSFILQTHIMREYPEAFTVS
P-----PYLNFRQLSPPLSSSKSDWLIGGAILTIQGIFVSVSFILQTHIMREYPEAFTVS
P-----PYLNFRQLSPPLSSSKSDWLIGGAILTIQGIFVSVSFILQAHIMREYPEAFTVS
P-----PYVNFRQFSPPLSSSNSDWLIGGALLTMQGIFVSVSFILQAHIMSVYPAAFRVS
P-----PYVNFRQFSPPLSSSNSDWLIGGALLTMQGIFVSVSFILQAHIMSVYPAAFRVS
P-----PYVNFRQFSPPLSSSNSDWLIGGALLTMQGIFVSVSFILQAHIMSVYPAAFRVS
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Transgenic Plants With Enhanced Agronomic Traits

Figure 1 of 4

```
P-----PYVNFRQFSPPLSSSNSDWLIGGALLTMQGIFVSVSFILQAHIMSVYPAAFRVS
P-----PYINFRQLSPPLSSSNSDWLIGGALLTIRDIFVSVSFILQAKIMSTYPAAFTVS
P-----PFPQLRQLLLPLSSSNSDWIIGGCLLAIKDTLVPVAFILQAHIMKLYPAPFTVS
P-----S------ISLRSQSPNHDWIIGAAFLAVEYFMVPLWYIVQTQIMREYPSEFTVV
S-----IS-----YNTLQFSPNLNWILGGFLCAGDSLLSSMWYIYQVSVTKKYPAVIVIV
D-------------NASIFVFDRDRVVGCIYLLGAVFVLSTNVVLQASTLAEFPAPISLS
D-------------NASIFVFDRDRVVGCMYLLGAVFILSSNVVLQASTLAEFPAPISLS
HP---------VFAHKTQSVGNGAWIRGTFLLILSCTTWSLWITLQVPLLIEYPNKLMAT
x-----xxxxxxxxxxxxxsxxxdwxxGxxxlxxxxxxxsxxxxlqxxxxxxyPxxxxxx

FWMCLFATIQSAMFALLKEP-DLQAWILPSPLQ-ISCSLYAG-IGIAVSFFIQSWCISER
FWMCLFATIQSAIFALLKEP-DLQAWILPSPLQ-ISCSLYAG-IGIAVSFFIQSWCISER
SFTCFFGVIQFLIIAAFFET-DLEHWKIHSGGE-LFTILYAGFVASGIAFSVQIWCIDRG
SYSCLFAVFQIVGISAYFER-DLESWKIKSGRE-MYALLYTGLVGSGMVFAIQIYVVERG
LVHNVCIAVVCAFVSLLAEKDNPKAWVIRFDIT-LITVVATGILNSGYY-VIHTWAVSHK
LVHNVCIAVVCAFVSLLAEKDNPKAWVIRFDIT-LITVVATGILNSGYY-VIHTWAVSHK
ILYILCISIVTSMIGLVVEKNNPSIWIIHFDIT-LFTIVTTGIITSVYY-VIHSWAIRHK
ILYILCISIVTSMIGLVVEKNNPSIWIIHFDIT-LFTIVTTGIITSVYY-VIHSWAIRHK
ILYILCISIVTSMIGLVVEKNNPSIWIIHFDIT-LFTIVTTGIITSVYY-VIHSWAIRHK
ILYILCISIVTSMIGLVVEKNNPSIWIIHFDIT-LFTIVTTGIITSVYY-VIHSWAIRHK
FLYTVCVSIVTSTIGLVVEKNNPSVWIIHFDIT-LITIVTMAIVTSVYY-VIHSWTVRHK
FLYTVCVSIVTSTIGLVVEKNNPSVWIIHFDIT-LITIVTMAIVTSVYY-VIHSWTVRHK
FLYTVCVSIVTSTIGLVVEKNNPSVWIIHFDIT-LITIVTMAIVTSVYY-VIHSWTVRHK
FLYTVCVSIVTSTIGLVVEKNNPSVWIIHFDIT-LITIVTMAIVTSVYY-VIHSWTVRHK
FLYIVSVSIVTSMIGLVVEKNNPSVWIIRFDIT-LITIVTMAIITSVYY-VIHSWTVRHK
FFYFLIASILTSLIGIVAEKNNPSIWIIHFDIT-LVCIVVGGIFNPGYY-AIHLWAVRNK
CYYSFGVSFWTGLATLFTEGSDLSAWKIKPNIA-LVSIVCSGLFGSCINNTIHTWALRIK
FFQVVFITIQTGVYALIVVR-DPSAWELKLDMG-LIVILYQAVAAIGIRYFLQTWSVQRA
AITALIGVLITMLVQLLQNQSGKVLTRSLISIGNLVGFSLLGGMVSGACVSFNGWAMKKR
AITSLIGVVITTMLQLLQNPNTKVVTRSLISISNLVGFSLLGGMVSGACVSFNGWAMKKR
AMQCLFSALQSFVVAVVVEK-DFTKWKLGLDIG-----LLAAFLGTGALMYLQAWCAEMS
xxxxxxxxxxxxxxlxxexxxxxxwxixxxix-lxxxxxxxxxxsxxxxxixxwxxxxx

GPLYCAMFNPLATVITALVAATFLEEKLYVGSLVGAIGVTVGLYIVLWGKAKDFDG----
GPLYCAMFNPLATVITALVAATFLEEKLYVGSLVGAIGVTVGLYIVLWGKAKDFDG----
GPVFVAVYQPVQTIAVAIMASIILGEQFYLGGIFGAILIIIGLYLVLWGKSEEKR-----
GPLFVSAYLPLQTLLAALLATFALGEHLYLGGLIGAILIICGLYLVVMGKSGRR------
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RPLYLAIFKPLSILIAVVMGTIFLNDSLYLGCLIGGILITLGFYVVMWGKA---------
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GPVYLAIFRPLSILIAVIMGAIFLGDSFYLGSLVGGILISLGFYTVMWGKA---------
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38-21(54973)B Mark ABAD 07/10/2008
Transgenic Plants With Enhanced Agronomic Traits
Figure 1 of 4

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VMPEKSQGQGDGDGATTQEKHGETNLTSQV-------------------------------
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Fig. 2 Plasmid map of pMON93039

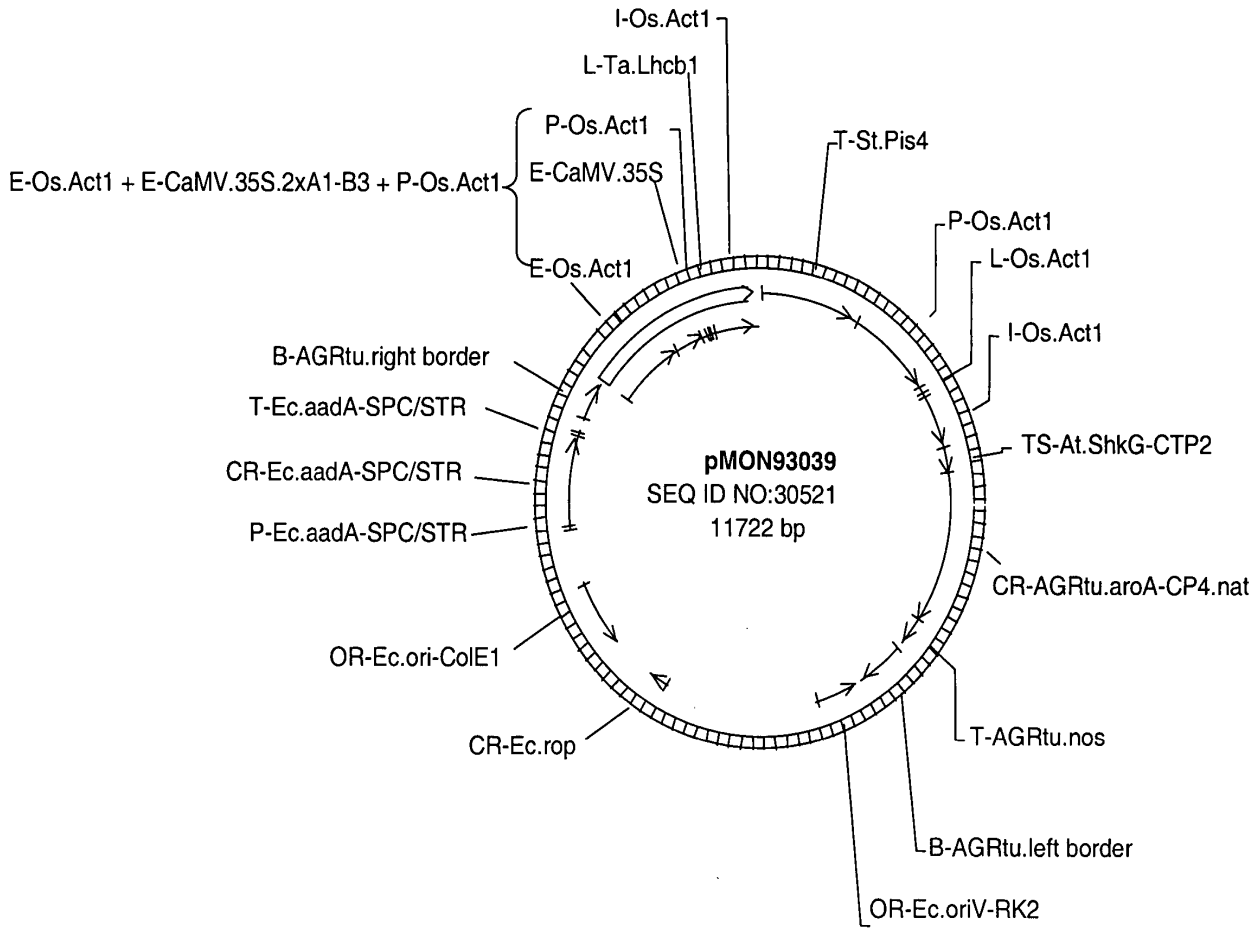
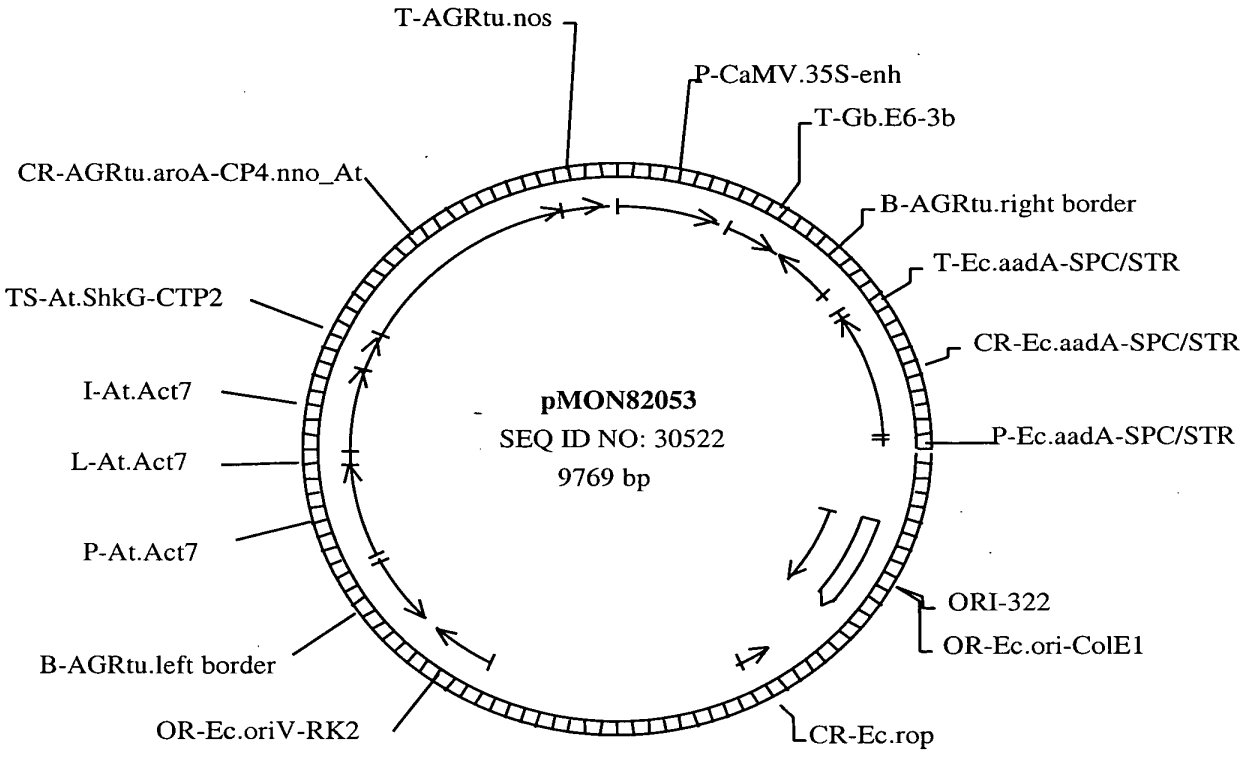


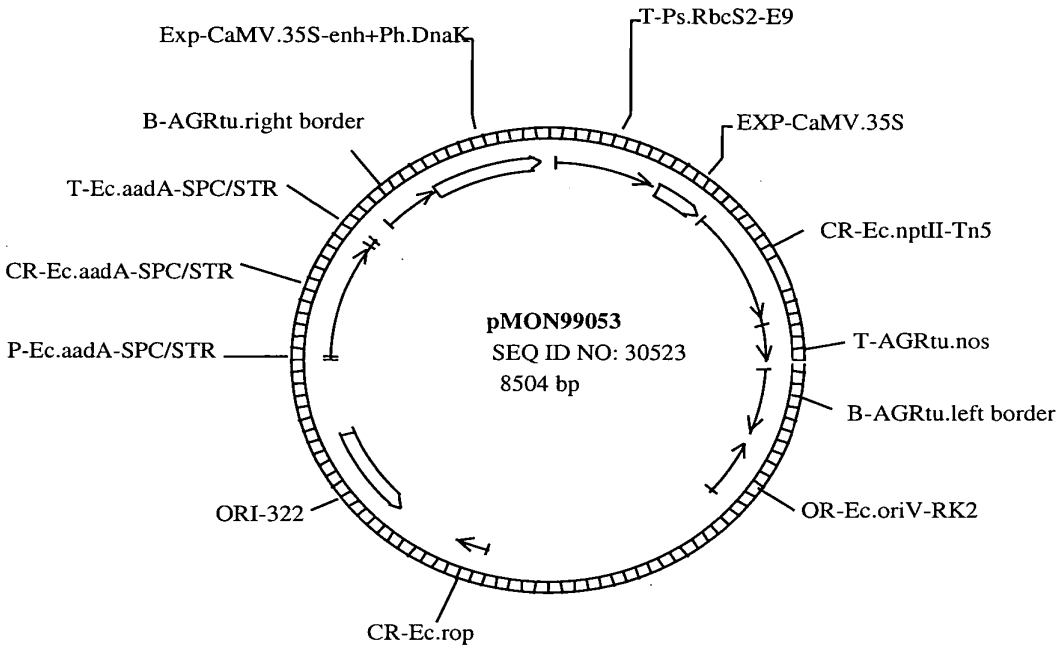
Fig. 3 Plasmid map of pMON82053



5

38-21(54973)B Mark ABAD 07/10/2008
Transgenic Plants With Enhanced Agronomic Traits
Figure 4 of 4

Fig. 4 Plasmid map of pMON99053



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[Continued on next page]

(54) Title: TRANSGENIC PLANTS WITH ENHANCED AGRONOMIC TRAITS

(57) Abstract: This invention provides recombinant DNA constructs, transgenic plant nuclei and cells with such recombinant DNA construct for expression of proteins that are useful for imparting enhanced agronomic trait(s) to transgenic crop plants. This invention also provides transgenic plants and progeny seed comprising the transgenic plant cells where the plants are selected for having an enhanced trait selected from the group of traits consisting of enhanced water use efficiency, enhanced cold tolerance, increased yield, enhanced nitrogen use efficiency, enhanced seed protein and enhanced seed oil. Also disclosed are methods for manufacturing transgenic seed and plants with enhanced traits.

WO 2009/009142 A3

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
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(88) Date of publication of the international search report:
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International application No.
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A. CLASSIFICATION OF SUBJECT MATTER

A01H 1/00(2006.01)i, C12N 15/82(2006.01)i, C12N 5/04(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC A01H 1/00, A01H 4/00, A01H 5/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean Utility models and applications for Utility models since 1975
Japanese Utility models and applications for Utility models since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKIPASS(KIPO internal) "recombinant DNA", "SEQ ID NO:309", " enhanced trait",

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KENNETH J. McDOWALL et al. 'The ams-1 and rne-3071 Temperature-Sensitive Mutations in the ams Gene Are in Close Proximity to Each Other and Cause Substitutions within a Domain That Resembles a Product of the Escherichia coli mre Locus.' Journal of Bacteriology. July 1993, pages 4245-4249. See the Abstract and figures 1-3	1
A	JP 15-310071 A(KUMHO PETROCHEM CO LTD) 5 NOV.2003 See the Claims 1-5 and figure 1	1
A	SWAINE L. CHEN et al. 'Identification of genes subject to postitive selection in uropathogenic strains of Escherichia coli: A comparative genomics approach.' PNAS. April 2006, Vol. 103, no.15, pages 5977-5982. See the Abstract and tables 1-2, figures 1-3, column 10	1

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
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"O" document referring to an oral disclosure, use, exhibition or other means
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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
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Date of the actual completion of the international search	Date of mailing of the international search report
06 FEBRUARY 2009 (06.02.2009)	**06 FEBRUARY 2009 (06.02.2009)**

Name and mailing address of the ISA/KR
Korean Intellectual Property Office
Government Complex-Daejeon, 139 Seonsa-ro, Seo-gu, Daejeon 302-701, Republic of Korea
Facsimile No. 82-42-472-7140

Authorized officer
CHO, Hyun Kyung
Telephone No. 82-42-481-5629

Form PCT/ISA/210 (second sheet) (July 2008)

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US2008/008557

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 2003-310071 A	05.11.2003	KR 2002042796 A	07.06.2002

Form PCT/ISA/210 (patent family annex) (July 2008)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2008/008557

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 2-16
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

: Claim 2 is too broad to make meaningful search regarding to the expression of constitutional elements (b) and (e). Since claims 3-16 are dependent on claim 2, they are broad too.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (July 2008)

Electronic Acknowledgement Receipt

EFS ID:	30809705
Application Number:	15678342
International Application Number:	
Confirmation Number:	1018
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Customer Number:	26191
Filer:	M. Angela Parsons/Shannon Glaeser
Filer Authorized By:	M. Angela Parsons
Attorney Docket Number:	38767-0048002
Receipt Date:	31-OCT-2017
Filing Date:	16-AUG-2017
Time Stamp:	11:41:53
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	Transmittal.pdf	69418 719ce6b264cae523da3fc16e5d4e155fecfece52	no	1

Warnings:

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2	Information Disclosure Statement (IDS) Form (SB08)	1449.pdf	124186 b486c4252892a6d826d101a52f85bcfc09e6d65a	no	1
Warnings:					
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3	Foreign Reference	WO2009009142.pdf	10181026 df03cfd423636871c18a5f628964a21becca336d	no	160
Warnings:					
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4	Non Patent Literature	LinCereghino_2006.pdf	11127312 be5f6ee9b8b2ace3900c804a61ee73638345e71b	no	15
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5	Non Patent Literature	Sievers_1978.pdf	560483 5dea8f7e4ce58a42c419ed524ad8c141d52d6a59	no	7
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New International Application Filed with the USPTO as a Receiving Office

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Attorney Docket No.: 38767-0048002

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First Named Inventor	:	Smita Shankar	Art Unit	:	1636
Serial No.	:	15/678,342	Examiner	:	Unknown
Filed	:	August 16, 2017	Conf. No.	:	1018
Title	:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST			

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Please consider the references listed on the enclosed PTO-1449 Form. Foreign patent documents and non-patent literature are enclosed. A copy of a Communication from a foreign Patent Office in a counterpart application also is enclosed.

This statement is being filed before the receipt of a first Office Action on the merits. Therefore, no fees are believed due. If this is incorrect, please apply any charges, or credits, to Deposit Account No. 06-1050, referencing the above-indicated Attorney Docket Number.

Please contact the undersigned if there are any questions regarding this statement.

Respectfully submitted,

Date: /November 29, 2017/

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Sheet 1 of 1

Substitute Disclosure Form	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No.	Application No.
Information Disclosure Statement by Applicant (Use several sheets if necessary)		38767-0048002	15/678,342
		Applicant: Impossible Foods Inc.	
(37 CFR §1.98(b))		Filing Date: August 16, 2017	Group Art Unit: 1636

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	--	--	--	--			

Foreign Patent Documents or Published Foreign Patent Applications								
Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
	1.	EP 2058398	05/13/2009	EP				
	2.	WO 2014/008729	01/16/2014	WIPO				

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
	3.	International Preliminary Report on Patentability in International Application No. PCT/US2016/031797, dated November 14, 2017, 7 pages

Examiner Signature	Date Considered
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Substitute Disclosure Form

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European Patent Office
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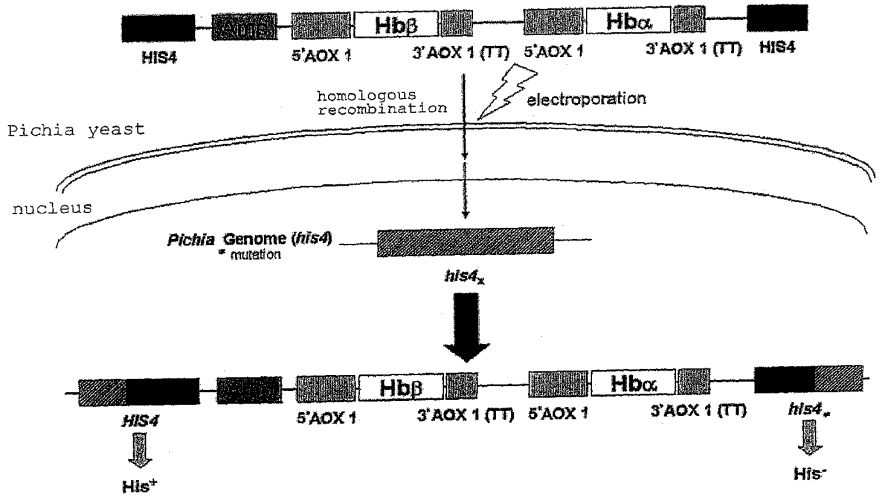
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(54) Production of recombinant human hemoglobin using pichia yeast

(57) The present invention provides a production method of a functional recombinant human hemoglobin, which comprises transforming a yeast of the genus Pichia with an expression vector containing a DNA encoding a human hemoglobin α-chain (Hbα), which is under regulation of a promoter functional in a yeast of the genus Pichia, and an expression vector containing a DNA encoding a human hemoglobin β-chain (Hbβ), which is under regulation of a promoter functional in a yeast of the genus Pichia, culturing the yeast in a medium, and recovering the human hemoglobin from the obtained culture. The present invention also provides an expression vector containing AOX1 promoter, DNA encoding Hbβ, terminator functional in a yeast of the genus Pichia, AOX1 promoter, DNA encoding Hbα and terminator functional in the yeast of the genus Pichia in this order from the upstream side in transcriptional direction.

FIG. 3



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Description

TECHNICAL FIELD OF THE INVENTION

[0001] The present invention relates to production of a recombinant human hemoglobin and an expression vector therefor. More particularly, the present invention relates to a production method of a human hemoglobin using a yeast of the genus Pichia, as well as an expression vector comprising in tandem an expression cassette containing a DNA encoding a human hemoglobin β-chain and an expression cassette containing a DNA encoding a human hemoglobin α-chain.

BACKGROUND OF THE INVENTION

[0002] Hemoglobin is a protein contained in red blood cells at a high concentration, and has a function to carry various gas molecules including oxygen. Structurally, it is a heterotetramer consisting of two α-chains and two β-chains.

[0003] As a preparation utilizing hemoglobin, a red blood cell substitute has been developed. There have so far been designed an intramolecular crosslinking type which is a hemoglobin modification form, a polymerization type, a polymer bond type, and hemoglobin endoplasmic reticulum in which hemoglobin is encapsulated in liposome. Particularly, there is a practical prospect of hemoglobin endoplasmic reticulum as a single administration oxygen transfusion for critical care, based on the property data of its shape, hemoglobin purity and the like, and the biological data of safety, in vivo retention, in vivo kinetics, oxygen-carrying ability and the like.

[0004] On the other hand, a preparation using a human blood-derived hemoglobin is associated with the risk of contamination with unknown virus and the like, and its use for human body is problematic in terms of safety. However, there is a possibility of provision of a safe hemoglobin preparation by a transformed microorganism using the gene recombination technique.

[0005] Until now, recombinant hemoglobin has been produced mainly using a system with Escherichia coli as a host cell. However, this method has problems in that (1) unless an enzyme (methionine aminopeptidase and the like) is coexpressed with hemoglobin and the like, addition of redundant first methionine to the N-terminal occurs, leading to a primary sequence different from that of a naturally occurring type, (2) aminolaevulinic acid needs to be added to a culture medium for the purpose of heme synthesis and the like.

[0006] Two cases have been reported so far as regards the production of recombinant hemoglobin using Saccharomyces cerevisiae (see Wagenbach, M. et al., Bio/Technol., 9: 57-61 (1991) and Coghlan, D. et al., Eur. J. Biochem., 207: 931-936 (1992)). In this system, since the first methionine is cleaved by an enzyme S. cerevisiae itself has, the primary sequence completely matches with that of a naturally occurring type, and addition of aminolaevulinic acid for heme synthesis is not necessary. However, this method is not entirely sufficient due to the problem of yield and the like, and therefore, an industrial production of recombinant human hemoglobin has not been practiced yet.

[0007] One of known industrial yeasts other than the genus Saccharomyces is a yeast of the genus Pichia. This yeast can grow with methanol as a sole carbon source, and when grown in methanol, an enzyme necessary for the treatment of methanol and its metabolism intermediate is disinhibited and expressed. Production of heterologous protein utilizing the methanol-utilizing pathway has been studied and applied to the production of albumin for blood preparations (see e.g., JP-A-6-22784). The expression amount thereof is known to be very high, and albumin of a 10 g order can be produced from 1 L of a medium.

[0008] However, there often exist cases where the expression efficiency of heterologous protein is low due to the combination (compatibility) of the object heterologous protein and a host cell. Particularly, in the case of a heterooligomer protein such as hemoglobin, since many conditions of balanced expression amounts of respective subunits, oligomer formation and the like need to be met, the desired high expression cannot be obtained easily. In fact, a yeast of the genus Pichia is used for industrial production of human protein in a very few cases.

[0009] An object of the present invention is to provide a means for producing a safe recombinant protein such as human hemoglobin or albumin, globulin and the like, which has sufficient function and activity similar to those of the naturally occurring type and is free of viral contamination and the like, easily in a large amount.

[0010] The present inventors have conducted intensive studies in an attempt to solve the above-mentioned problems and succeeded in recovering various proteins or peptides such as a human hemoglobin and the like, particularly a heterooligomer protein and the like, in a high yield by transforming Pichia pastoris, which is one of the yeasts of the genus Pichia, with an expression vector containing a DNA encoding plural proteins or peptide chains, for example, an expression vector containing a DNA encoding α-chain and β-chain of human hemoglobin, and cultivating the obtained transformant in a medium, which resulted in the completion of the present invention.

SUMMARY OF THE INVENTION

[0011] Accordingly, the present invention provides

(1) a production method of a functional recombinant human hemoglobin, which comprises transforming a yeast of the genus Pichia with an expression vector containing a DNA encoding a human hemoglobin α-chain, which is under regulation of a promoter functional in a yeast of the genus Pichia, and an expression vector containing a DNA encoding a human hemoglobin β-chain, which is under regulation of a promoter functional in a yeast of the genus Pichia, culturing the yeast in a medium, and recovering the human hemoglobin from the obtained culture;
(2) the method of the above-mentioned (1), wherein the DNA encoding the human hemoglobin α-chain and the DNA encoding the human hemoglobin β-chain are on the same vector;
(3) the method of the above-mentioned (2), wherein the DNA encoding the human hemoglobin β-chain is at the upstream side in the transcriptional direction;
(4) the method of any of the above-mentioned (1) - (3), wherein the DNA encoding the human hemoglobin α-chain and the DNA encoding the human hemoglobin β-chain are under regulation of different promoters;
(5) the method of any of the above-mentioned (1) - (4), wherein the DNA encoding the human hemoglobin α-chain and the DNA encoding the human hemoglobin β-chain are under regulation of an alcoholoxidase 1 promoter;
(6) the method of the above-mentioned (5), wherein the transformed yeast of the genus Pichia is cultivated in a liquid medium requiring methanol as a single carbon source;
(7) the method of the above-mentioned (6), wherein the culture is a feeding culture;
(8) the method of any of the above-mentioned (1) - (7), wherein the yeast of the genus Pichia is Pichia pastoris; and
(9) an expression vector comprising an alcoholoxidase 1 promoter, a DNA encoding the human hemoglobin β-chain, a terminator functional in a yeast of the genus Pichia, an alcoholoxidase 1 promoter, a DNA encoding a human hemoglobin α-chain and a terminator functional in a yeast of the genus Pichia in this order from the upstream side in the transcriptional direction.
The present invention also provides
(10) a method of producing an expression vector comprising a DNA encoding plural proteins, which is under control of a promoter functional in a yeast of the genus Pichia, wherein the vector and a DNA encoding one of the plural proteins are ligated without a dephosphorylation treatment of the vector;
(11) the method of the above-mentioned (10), wherein (i) an expression vector containing a DNA encoding one or more proteins, which is under control of a promoter functional in a yeast of the genus Pichia, wherein the expression vector is digested with a restriction enzyme capable of cleavage thereof other than the inside of the promoter and the DNA and the both ends produced thereby are not dephosphorylated is provided, (ii) a DNA encoding other protein, which is under control of a promoter functional in a yeast of the genus Pichia, wherein the both ends are complementary to the end of the expression vector of the above-mentioned (i) is provided, and (iii) the expression vector of the above-mentioned (i) and the DNA of the above-mentioned (ii) are ligated;
(12) a production method of a recombinant protein, which comprises culturing, in a medium, a yeast of the genus Pichia transformed with an expression vector obtained by the method of the above-mentioned (10) or (11), and recovering the plural proteins from the obtained culture;
(13) the method of the above-mentioned (12), wherein the plural proteins are polymerized in the culture to form a functional protein complex; and the like.

[0012] Since the human hemoglobin provided by the method of the present invention is produced using a yeast of the genus Pichia as a host by the gene recombination technique, it is free of a risk of contamination with an unknown virus and the like, which is a problem specific to blood-derived preparations, and can be used safely for human body and the like. Using a yeast of the genus Pichia, moreover, a human hemoglobin can be produced in a large amount, which has similar function and properties as the naturally occurring type. Accordingly, administration thereof to a human body etc. causes less influence such as side effects and the like.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013]

Fig. 1 is an explanatory drawing of the principle of connection between hemoglobin α-chain expression cassette and β-chain expression cassette.
Fig. 2 is an explanatory drawing of the outline of the procedures for preparing hemoglobin in the Examples.
Fig. 3 is an explanatory drawing of the recombination method of hemoglobin with Pichia chromosome in the Examples.

DETAILED DESCRIPTION OF THE INVENTION

[0014] As a DNA encoding the human hemoglobin α-chain to be used in the present invention (hereinafter sometimes to be abbreviated as "Hbα"), a DNA containing a base sequence encoding an amino acid sequence the same or substantially the same as the amino acid sequence shown in SEQ ID NO: 2, and as a DNA encoding a human hemoglobin β-chain (hereinafter sometimes to be abbreviated as "Hbβ"), a DNA containing a base sequence encoding an amino acid sequence the same or substantially the same as the amino acid sequence shown in SEQ ID NO: 4 can be mentioned.

[0015] As the amino acid sequence the same or substantially the same as the amino acid sequence shown in SEQ ID NO: 2 (or SEQ ID NO: 4), an amino acid sequence having a homology of not less than about 80%, preferably not less than about 90%, more preferably not less than about 95%, particularly preferably not less than about 97% with the amino acid sequence shown in SEQ ID NO: 2 (or SEQ ID NO: 4) and the like can be mentioned. As used herein, by the "homology" is meant the proportion (%) of the same amino acid and similar amino acid residues relative to the total overlapping amino acid residues in the optimal alignment when two amino acid sequences are aligned using a mathematical algorithm known in the art (preferably, the algorithm is capable of considering introduction of a gap into one or both of the sequences for the optimal alignment). The "similar amino acid" means an amino acid similar in the physicochemical properties. For example, amino acids classified in the same group such as aromatic amino acids (Phe, Trp, Tyr), aliphatic amino acids (Ala, Leu, Ile, Val), polar amino acids (Gln, Asn), basic amino acids (Lys, Arg, His), acidic amino acids (Glu, Asp), amino acids (Ser, Thr) having a hydroxyl group, amino acids (Gly, Ala, Ser, Thr, Met) with small side chain and the like can be mentioned. It is predicted that substitution with such similar amino acids will not alter protein phenotypes (namely, preservative amino acid substitution). Specific examples of preservative amino acid substitution are well known in the art and are described in various literatures (see e.g., Bowie et al., Science, 247: 1306-1310 (1990)).

[0016] The homology of the amino acid sequence in the present specification can be calculated using homology calculation algorithm NCBI BLAST (National Center for Biotechnology Information Basic Local Alignment Search Tool) under the following conditions (expectancy=10; allowing gap; matrix=BLOSUM62; filtering=OFF). Other algorithms for determining the homology of the amino acid sequence include, for example, the algorithm described in Karlin et al., Proc. Natl. Acad. Sci. USA, 90: 5873-5877 (1993) [this algorithm is incorporated in the NBLAST and XBLAST program (version 2.0) (Altschul et al., Nucleic Acids Res., 25: 3389-3402 (1997))], the algorithm described in Needleman et al., J. Mol. Biol., 48: 444-453 (1970) [this algorithm is incorporated in the GAP program in the GCG software package], the algorithm described in Myers and Miller, CABIOS, 4: 11-17 (1988) [this algorithm is incorporated in the ALIGN program (version 2.0) which is a part of the CGC sequence alignment software package], the algorithm described in Pearson et al., Proc. Natl. Acad. Sci. USA, 85: 2444-2448 (1988) [this algorithm is incorporated in the FASTA program in the GCG software package] and the like, and they can also be used preferably.

[0017] More preferably, an amino acid sequence substantially same as the amino acid sequence shown in SEQ ID NO: 2 (or SEQ ID NO: 4) has homology of not less than about 80%, preferably not less than about 90%, more preferably not less than about 95%, particularly preferably not less than about 97%, to the amino acid sequence shown in SEQ ID NO: 2 (or SEQ ID NO: 4).

[0018] A protein containing an amino acid sequence substantially same as the amino acid sequence shown in SEQ ID NO: 2 (or SEQ ID NO: 4) means a protein containing an amino acid sequence substantially same as the aforementioned amino acid sequence shown in SEQ ID NO: 2 (or SEQ ID NO: 4) and having a substantially equivalent activity to that of the protein containing the amino acid sequence shown in SEQ ID NO: 2 (or SEQ ID NO: 4).

[0019] The substantially equivalent activity includes, for example, the physiological activity of human hemoglobin, for example, an activity to carry a gas molecule (e.g., oxygen and the like), and the like. The "substantially equivalent" means that the activities are qualitatively same. Therefore, the activity of oxygen-carrying ability and the like is preferably equivalent (e.g., about 0.5- to about 2-fold). However, quantitative elements such as the level of the activity, molecular weight of the protein and the like may be different.

[0020] The activity of oxygen-carrying ability and the like (e.g., autoxidation rate constant, oxygen affinity, cooperativity and the like) can be measured according to, but not limited to, a method known per se, for example, the method described in JP-A-2004-500871 and the like.

[0021] In addition, Hbα (or Hbβ) to be used in the present invention includes, for example, a protein containing (1) an amino acid sequence wherein one or more (preferably about 1 - 30, more preferably about 1 - 10, particularly preferably 1 - several (2, 3, 4 or 5)) amino acids are deleted from the amino acid sequence shown in SEQ ID NO: 2 (or SEQ ID NO: 4), (2) an amino acid sequence wherein one or more (preferably about 1 - 30, more preferably about 1 - 10, particularly preferably 1 - several (2, 3, 4 or 5)) amino acids are added to the amino acid sequence shown in SEQ ID NO: 2 (or SEQ ID NO: 4), (3) an amino acid sequence wherein one or more (preferably about 1 - 30, more preferably about 1 - 10, particularly preferably 1 - several (2, 3, 4 or 5)) amino acids are inserted in the amino acid sequence shown in SEQ ID NO: 2 (or SEQ ID NO: 4), (4) an amino acid sequence wherein one or more (preferably about 1 - 30, more preferably about 1 - 10, particularly preferably 1 - several (2, 3, 4 or 5)) amino acids are substituted by other amino acids in the

amino acid sequence shown in SEQ ID NO: 2 (or SEQ ID NO: 4), or (5) a combination of these, and the like.

[0022] When the amino acid sequence is inserted, deleted or substituted as mentioned above, the site of the insertion, deletion or substitution is not particularly limited as long as the activity of protein is maintained.

[0023] Human hemoglobin in the present invention is used to mean not only adult hemoglobin (HbA1) but encompass HbA2 wherein α-chain and δ-chain are bonded, fetal hemoglobin (HbF) wherein α-chain and γ-chain are bonded, and the like.

[0024] For the production of HbA2 and HbF, therefore, a DNA encoding δ-chain and γ-chain instead of β-chain is used. The amino acid sequence and base sequence of δ-chain are registered, for example, in GenBank accession Nos. NP_000510 and NM_000519. On the other hand, the amino acid sequence and base sequence of γ-chain are registered, for example, in GenBank accession Nos. NP_000550 and NM_000559.

[0025] Examples of the DNA encoding Hbα (or Hbβ) include human genomic DNA, cDNA derived from Hbα (and/or Hbβ)-producing cells (e.g., red blood cell and the like) of adult, synthetic DNA and the like. The genomic DNA and cDNA encoding Hbα (or Hbβ) can also be directly amplified by Polymerase Chain Reaction (hereinafter to be abbreviated as "PCR method") and Reverse Transcriptase-PCR (hereinafter to be abbreviated as "RT-PCR method"), using a genomic DNA fraction and total RNA or an mRNA fraction prepared from a producing-cell or tissue (e.g., blood and the like) thereof as respective templates. Alternatively, the genomic DNA and cDNA encoding Hbα (or Hbβ) can be respectively cloned by colony or plaque hybridization method, PCR method and the like, from a genomic DNA library and cDNA library prepared by inserting genomic DNA and total RNA or an mRNA fraction prepared from the above-mentioned cell or tissue into suitable vectors. The vector to be used for the library may be any such as bacteriophage, plasmid, cosmid, phagemid and the like.

[0026] Examples of the DNA encoding Hbα (or Hbβ) include DNA containing the base sequence shown in SEQ ID NO: 1 (or SEQ ID NO: 3), DNA having a base sequence hybridizing with the base sequence shown in SEQ ID NO: 1 (or SEQ ID NO: 3) under stringent conditions, and encoding a protein having substantially equivalent activity (e.g., oxygen-carrying ability and the like) to that of the aforementioned Hbα (or Hbβ) and the like.

[0027] As the DNA capable of hybridizing to the base sequence shown in SEQ ID NO: 1 (or SEQ ID NO: 3) under stringent conditions, for example, DNA containing a base sequence having homology of not less than about 70%, preferably not less than about 80%, more preferably not less than about 90%, particularly preferably not less than about 95%, to the base sequence shown in SEQ ID NO: 1 (or SEQ ID NO: 3) and the like are used.

[0028] The homology of the base sequence in the present specification can be calculated using a homology calculation algorithm NCBI BLAST (National Center for Biotechnology Information Basic Local Alignment Search Tool) under the following conditions (expectancy=10; allowing gap; filtering=ON; match score=1; mismatch score=-3). Preferable examples of other algorithms usable for determining homology of the base sequence include the above-mentioned homology calculation algorithms for amino acid sequence.

[0029] Hybridization can be performed according to a method known per se or a method according to the method, for example, the method described in Molecular Cloning, ver. 2 (J. Sambrook et al., Cold Spring Harbor Lab. Press, 1989) and the like. When a commercially available library is used, moreover, hybridization can be performed according to the method described in the attached instruction manual. Preferably, hybridization can be performed under stringent conditions.

[0030] The high stringent conditions include, for example, a hybridization reaction at 45°C in 6×SSC (sodium chloride/sodium citrate), and washing one or more times at 65°C in 0.2×SSC/0.1% SDS and the like.

[0031] Those of ordinary skill in the art can easily adjust to the desired stringency by appropriately changing the salt concentration of hybridization solution, temperature of hybridization reaction, probe concentration, length of probe, number of mismatches, hybridization reaction time, salt concentration of washing solution, temperature of washing and the like.

[0032] A DNA encoding Hbα (or Hbβ) can be cloned by amplifying by PCR method using a synthetic DNA primer having a part of the base sequence encoding Hbα (or Hbβ), or hybridizing DNA incorporated into a suitable expression vector to with a labeled DNA fragment or synthetic DNA encoding a part or full region of Hbα (or Hbβ).

[0033] A DNA encoding Hbα (or Hbβ) can be obtained from human genomic DNA or RNA (cDNA) as mentioned above. It is also possible to construct a DNA encoding the full-length Hbα (or Hbβ) by chemically synthesizing a DNA chain or connecting a synthesized, partly overlapping oligo DNA short chain by PCR method. The advantage of constructing a full-length DNA by a combination of chemical synthesis and PCR method is that the codon to be used can be designed over the full length of the gene in accordance with the host to be introduced into the gene. Plural codons encoding the same amino acid are not uniformly used and the frequency of use varies depending on the species. In general, a codon contained in a gene highly expressing in a certain species contains a large amount of codon frequently used in the species. On the other hand, in many of the genes with a low expression amount, the presence of a codon used less frequently often becomes a bottleneck in the high expression. There are many reports on the examples wherein, in the expression of a heterologous gene, the expression amount of the heterologous protein was increased by substituting the gene sequence with a codon highly frequently used in the host organism, and therefore, such alteration

of the codon to be used is expected to be effective for increasing the expression amount of a heterologous gene.

[0034] For the above-mentioned reasons, a DNA encoding Hbα (or Hbβ) can be altered to a codon more suitable for a yeast of the genus Pichia into which the DNA is to be introduced (that is, a codon highly frequently used in the host organism). The frequency of use of the codon in each yeast of the genus Pichia is defined by the ratio of use of each codon to all the genes present on the genomic sequence of the host, for example, by the number of use per 1000 codons. In addition, the frequency of use of codon can also be approximately calculated from plural representative gene sequences in the case of a biological entity for which the complete genomic sequences have not been identified. As for the data of frequent use of codon in yeast of the genus Pichia to be genetically modified, for example, the genetic code usage frequency database (http://www.kazusa.or.jp/codon/index.html) disclosed in the homepage of Kazusa DNA Research Institute can be used. Alternatively, a document indicating the frequency of use of codon in each yeast of the genus Pichia may be referred to, or the data of frequency of use of codon in the yeast of the genus Pichia to be used may be constructed by oneself. By referring to the obtained data and the gene sequence to be introduced, the codon used in the gene sequence, which shows a low frequency of use in the host yeast, can be substituted to a codon encoding the same amino acid and showing a high frequency of use.

[0035] Another advantage of constructing a DNA encoding the full-length Hbα (or Hbβ) by chemically synthesizing a DNA chain, or connecting a synthesized, partly overlapping oligo DNA short chain by PCR method is easy construction of an expression vector thereafter. For example, in the below-mentioned Examples, an expression cassette of Hbβ and an expression cassette of Hbα are connected in tandem, utilizing the complementarity of sticky end produced by digestion with restriction enzymes BamH I and Bgl II (see Fig. 1. Utilizing the complementarity of sticky end, multicopy expression cassette can be repeatedly integrated in tandem into a vector). Therefore, the DNA encoding Hbα and the DNA encoding Hbβ are preferably free of a BamH I recognition site. For example, Hbβ cDNA registered as GenBank accession No. NM_000518 in the NCBI database has a BamH I recognition site (GGATCC) at the 347th - 352nd positions. In the base sequence shown in SEQ ID NO: 3, the base sequence of the corresponding part (base Nos. 297 - 302) is TGACCC, where a mutation has been introduced to not contain a BamH I recognition site. In the below-mentioned Examples, moreover, an EcoR I recognition sequence is added to both ends of a DNA encoding Hbα and a DNA encoding Hbβ to construct an expression cassette by inserting a DNA encoding Hbα and a DNA encoding Hbβ between a promoter and a terminator functional in a yeast of the genus Pichia (see Fig. 2). Therefore, a DNA encoding Hbα and a DNA encoding Hbβ is preferably free of a EcoR I recognition site. For example, Hbβ cDNA registered as GenBank accession No. NM_000518 in the NCBI database has an EcoR I recognition site (GAATTC) at the 414th to 419th positions. In the base sequence shown in SEQ ID NO: 3, the base sequence of the corresponding part (base Nos. 370 - 375) is GAGTTT, where a mutation has been introduced to not contain an EcoR I recognition site. When a restriction enzyme recognition site in a protein-coding sequence is to be altered, it is desirable to substitute a base, thereby to not change the amino acid sequence of Hbα (or Hbβ) utilizing degeneration of the codon.

[0036] A DNA cloned as mentioned above can be used as it is depending on the object or used after digestion with a restriction enzyme or adding a linker. The DNA may have ATG as a translation initiation codon at the 5' end side thereof and TAA, TGA or TAG as a translation stop codon at the 3' end side thereof. These translation initiation codon and translation stop codon can be added using a suitable synthetic DNA adapter.

[0037] An expression vector containing a DNA encoding Hbα (or Hbβ) can be produced, for example, by ligating a DNA fragment encoding Hbα (or Hbβ) cloned as mentioned above to a downstream of a promoter in a suitable expression vector using a restriction enzyme and a DNA ligase.

[0038] The vector to be used is not particularly limited as long as it can be maintained stably by autonomous replication in a fungus body of yeast of the genus Pichia or integration into a yeast genome. Examples of the autonomously-replicable vector include YEp vector, YRp vector, YCp vector and the like. In addition, examples of the vector to be integrated into a yeast genome include YIp vector and YRp vector.

[0039] Examples of the promoter functional in the yeast of the genus Pichia include promoters derived from a yeast, such as PHO5 promoter, PGK promoter, GAP promoter, ADH promoter derived from S. cerevisiae and the like, alcohol oxidase (AOX) 1 promoter, AOX2 promoter, dihydroxyacetone synthase promoter, P40 promoter, ADH promoter, folic acid dehydrogenase promoter derived from P. pastoris and the like. In addition, the above-mentioned promoter derived from a yeast may be a mutant promoter modified to further improve the gene expression efficiency, for example, mutant AOX2 (mAOX2) promoter [Ohi et al., Mol. Gen. Genet., 243, 489-499 (1994); JP-A-4-299984] and the like. Preferably, the promoter is a promoter of an enzyme gene necessary for treating methanol or a metabolic intermediate thereof, in order to use a methanol-metabolizing system in the yeast of the genus Pichia, such as AOX1 promoter, mAOX2 promoter and the like, more preferably AOX1 promoter.

[0040] The expression vector containing the DNA encoding Hbα (or Hbβ) of the present invention preferably further contains transcription terminator sequence (terminator) functional in a yeast of the genus Pichia (e.g., AOX1 terminator etc.), enhancer sequence, selection marker gene usable for selecting yeast (auxotrophic gene, for example, HIS4, LEU2, ARG4 and URA3 gene derived from P. pastoris or S. cerevisiae, and the like, or antibiotic resistance gene, for example, resistance gene to cycloheximide, G-418, chloramphenicol, bleomycin, hygromycin etc., and the like) and the like, and

when desired, may contain replicable unit functional in yeast. For preparation of the vector in a large amount, moreover, the vector more preferably contains a replicable unit functional in Escherichia coli and a selection marker gene usable for selecting Escherichia coli (e.g., resistance gene to ampicillin and tetracycline etc.).

[0041] When the expression vector is of a type incorporated into a yeast genome, the vector preferably further contains a sequence homologous to a yeast genome necessary for homologous recombination. As such homology sequence, the aforementioned auxotrophic gene sequence can be mentioned. Accordingly, in one preferable embodiment, the expression vector of the present invention is one wherein an expression cassette of the above-mentioned Hbα (or Hbβ) is inserted in an auxotrophic gene (in the present specification, the "expression cassette" means a unit enabling gene expression, whose minimal unit is a protein-coding sequence configured under regulation of a promoter, with preference given to a unit comprising promoter-protein-coding region-terminator) (see Fig. 3).

[0042] An expression cassette of Hbα and an expression cassette of Hbβ may be carried on individual vectors or the same vector. In the former case, two vectors desirably contain different selection marker genes. However, in a preferable embodiment of the present invention, the expression cassette of Hbα and the expression cassette of Hbβ are configured on the same vector. In this case, the expression cassette of Hbα and the expression cassette of Hbβ may be configured in any order. For example, the expression cassettes may be configured head-to-head or tail-to-tail on an opposite chain. Preferably, they are configured in the same transcriptional direction, more preferably, the expression cassettes are configured in the order of the expression cassette of Hbβ and the expression cassette of Hbα, from the upstream side in the transcriptional direction. When the DNA encoding Hbα and the DNA encoding Hbβ are transcribed in the same direction, the transcription may be performed monocistronically from the same promoter or dicistronically from separate promoters. In the case of monocistronic transcription, the expression vector does not contain a terminator at the 3' side of the coding sequence in the upstream. In addition, an internal ribosome entry site (IRES) is preferably contained at the 5' side of the coding sequence in the downstream. However, in a preferable embodiment of the present invention, the DNA encoding Hbα and the DNA encoding Hbβ are transcribed dicistronically from separate promoters.

[0043] In a particularly preferable embodiment of the present invention, an expression vector containing AOX1 promoter, DNA encoding Hbβ, terminator functional in a yeast of the genus Pichia (preferably AOX1 terminator), AOX1 promoter, DNA encoding Hbα and terminator functional in the yeast of the genus Pichia (preferably AOX1 terminator) in this order from the upstream side in transcriptional direction is used.

[0044] For construction of an expression vector comprising the above-mentioned expression cassette of Hbβ and the expression cassette of Hbα ligated in tandem, generally, a site immediately upstream (when expression cassette of Hbα is contained) of the promoter of the expression vector containing one of the expression cassettes or a site immediately downstream (when expression cassette of Hbβ is contained) of the terminator thereof is cleaved with a suitable restriction enzyme (e.g., in Fig. 2, Hbβ-pAO815 is cleaved with BamH I) to linearize the vector, and ligated using the other expression cassette cleaved by treating with a restriction enzyme providing the same sticky end (or blunt end) (e.g., in Fig. 2, expression cassette of Hbα is cleaved out from Hbα-pAO815 by double digestion with BamH I and Bgl II) and a DNA ligase. At this time, in general, a dephosphorylation treatment of the end (e.g., calf intestine alkaline phosphatase (CIAP) treatment) is performed to prevent self-ligation on the vector side. In some cases, the total ligation efficiency can be enhanced when the dephosphorylation treatment is not applied.

[0045] The expression vector obtained as mentioned above can be introduced into the fungus body of the target yeast of the genus Pichia using, for example, a known transformation technique such as competent cell method, protoplast method, calcium phosphate coprecipitation method, polyethylene glycol method, lithium method, electroporation method, microinjection method, liposome fusion method, particle gun method and the like.

[0046] While the yeast of the genus Pichia to be used in the present invention is not particularly limited, for example, P. pastoris, Pichia acaciae, Pichia angusta, Pichia anomala, Pichia capsulata, Pichia ciferrii, Pichia etchellsii, Pichia fabianii, Pichia farinosa, Pichia guilliermondii, Pichia inositovora, Pichia jadinii, Pichia methanolica, Pichia norvegensis, Pichia ofunaensis, Pichia pinus and the like can be used. Preferred is P. pastoris, particularly, auxotrophic mutant P. pastoris strain (e.g., P. pastoris GTS115 strain ($HIS4^-$) [NNRL Y-15851], P. pastoris GS190 strain ($ARG4^-$) [NNRLY-18014), P. pastoris PPF1 ($HIS4^-$, $URA4^-$) [NNRL Y-18017] and the like).

[0047] By cultivating the transformed yeast of the genus Pichia by a method generally used in the art, a functional human hemoglobin can be produced. The medium to be used needs to contain at least a carbon source and an inorganic or organic nitrogen source necessary for the growth of the host cell. Examples of the carbon source include methanol, glycerol, glucose, sucrose, dextran, soluble starch and the like. In addition, examples of the inorganic or organic nitrogen source include ammonium salts, nitrate salts, amino acid, corn steep liquor, peptone, casein, meat extract, yeast extract, soybean cake, potato extract and the like. When desired, moreover, other nutrients, for example, inorganic salts such as calcium chloride, sodium dihydrogenphosphate, magnesium chloride and the like, vitamins such as biotin and the like, antibiotic and the like can be added.

[0048] Examples of the medium to be used include conventional natural medium (e.g., YPD medium, YPM medium, YPG medium etc.) and synthetic medium. As the pH and culture temperature of the medium, those suitable for the growth of yeast and production of human hemoglobin are employed. For example, pH of about 5 - about 8, culture

temperature of about 20°C - about 30°C are preferable. In addition, aeration and agitation are performed as necessary. The culture is generally performed for about 48 - about 120 hr.

[0049] For example, when a promoter whose expression is induced by methanol, such as AOX1 promoter, mAOX2 promoter and the like, is used as a promoter functional in the fungus body of a yeast of the genus Pichia, a method of liquid aeration-agitation culture using natural medium controlled to pH about 6.0, which contains glycerol as a carbon source for the growth of fungus body and methanol as an Hbα and Hbβ expression inducer is most preferable. When the expression of Hbα and Hbβ is not preferable for the growth of fungus body, a method including first increasing the amount of fungus body with a carbon source other than methanol, and inducing the expression of Hbα and Hbβ by addition of methanol is more preferable. In a culture in a jarfermenter, moreover, a high density culture method is suitable for the production of human hemoglobin. The culture may be performed by any of batch culture, feeding culture and continuous culture, with preference given to feeding culture method. That is, for a certain period, a method including culturing the host fungus body in a medium (initial medium) containing a carbon energy source suitable for the growth (e.g., glucose etc.) and/or a nutrient source, and confining human hemoglobin in the system until completion of the culture while additionally supplying a substrate controlling the expression of Hbα and Hbβ (that is, methanol) to the medium from a certain point in time according to the situation can be used (see e.g., JP-A-3-83595).

[0050] Human hemoglobin produced in the culture can be collected in the yeast fungus body by applying the culture after completion of cultivation to centrifugation and/or filtration, and subsequently isolated and purified from the fungus body according to a method known per se. As such method, a method utilizing the solubility such as salting out, solvent precipitation and the like; a method mainly utilizing difference in the molecular weight such as dialysis, ultrafiltration, gel filtration method, SDS-polyacrylamide gel electrophoresis and the like; a method utilizing difference in the electric charge such as ion exchange chromatography and the like; a method utilizing specific affinity such as affinity chromatography and the like; a method utilizing difference in hydrophobicity such as reversed-phase high performance liquid chromatography and the like; a method utilizing difference in the isoelectric point such as isoelectric focusing and the like; and the like can be used. These methods can be appropriately combined.

[0051] Examples of a method for confirming the isolated and purified human hemoglobin include known Western blotting method, activity measurement method and the like. In addition, the structure of the purified hemoglobin can be clarified by amino acid analysis, N-terminal amino acid sequence, primary structure analysis and the like. Human hemoglobin obtained in this way has the same primary structure (that is, removal by cleavage of first methionine) and higher-order structure as those of the naturally occurring type, and shows the physiological activity of the same quality as the naturally occurring type.

[0052] The recombinant human hemoglobin produced in the present invention can be administered to the living body, for example, as a red blood cell substitute. For administration as a red blood cell substitute, hemoglobin can be administered by encapsulating in liposome or dispersing in emulsion. The methods of preparation making and method of administration to human are described in, for example, JP-A-2004-307404, JP-A-2006-104069, JP-A-2002-161047, JP-A-2001-348341, JP-A-08-003063, JP-A-08-003062, JP-A-07-017874, JP-A-06-321802, JP-A-06-072892 and the like.

SEQUENCE LISTING FREE TEXT

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primer.
SEQ ID NO: 7
primer.
SEQ ID NO: 8
primer.
SEQ ID NO: 9
primer.
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primer.
SEQ ID NO: 11
primer.
SEQ ID NO: 12
primer.
SEQ ID NO: 13
Hbα cDNA having EcoR I site at 5' and 3'-ends.

SEQ ID NO: 14
Hbβα cDNA having EcoR I site at 5' and 3'-ends.
SEQ ID NO: 15
primer.
SEQ ID NO: 16
primer.
SEQ ID NO: 17
primer.
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primer.
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primer.

[0054] The present invention is explained in more detail in the following by referring to Examples, which are mere exemplifications and do not limit the scope of the present invention.

EXAMPLES

(1) amplification of hemoglobin gene

[0055] Using plasmid pBEX transfected with human hemoglobin gene (hereinafter referred to as "pBEX-Hb". Available from Tadayuki Uno (Graduate School and School of Pharmaceutical Sciences, Osaka University), a coinventor of the present application) as a template, and Hbα-sense primer of SEQ ID NO: 5 and Hbα-antisense primer of SEQ ID NO: 6 as synthetic primers of the DNA sequence of hemoglobin α-chain, and Hbβ-sense primer of SEQ ID NO: 7 and Hbβ-antisense primer of SEQ ID NO: 8 as synthetic primers of the DNA sequence of hemoglobin β-chain, respectively, mutation of EcoR I restriction enzyme site, which would become a obstruction of subsequent manipulation, was performed (QuikChange XL Site-Directed Mutagenesis Kit, Stratagene). As for mutation reaction conditions, DNA was treated for 30 sec at 95°C, after which a 12-cycle reaction of denaturation (95°C, 30 sec), annealing (55°C, 1 min) and extension (68°C, 10 min) was performed. After the reaction, the template plasmid was digested by Dpn I, and each of obtained pBEX-Hb(αΔ) and pBEX-Hb(βΔ) were transfected into XL-10-Gold ultracompetent cells to perform transformation. The transformants, which were transfected with the objective plasmids pBEX-Hb(αΔ) or pBEX-Hb(βΔ), were screened in ampicillin-added medium, and the plasmids were purified from the obtained transformants (QIAprep Spin Miniprep Kit, manufactured by QIAGEN). The plasmids were digested with EcoR I (manufactured by Takara Shuzo Co., Ltd.), and the mutation was confirmed.

[0056] Using the obtained pBEX-Hb(αΔ) and pBEX-Hb(βΔ) as templates, and Hbα-sense primer of SEQ ID NO: 9 and Hbα-antisense primer of SEQ ID NO: 10 as synthetic primers of the DNA sequence of hemoglobin α-chain, and Hbβ-sense primer of SEQ ID NO: 11 and Hbβ-antisense primer of SEQ ID NO: 12 as synthetic primers of the DNA sequence of hemoglobin β-chain, PCR was performed using a polymerase (KOD-plus-, manufactured by Toyobo Co., Ltd.). As for PCR reaction conditions, DNA was treated for 2 min at 94°C, after which a 30-cycle reaction of denaturation (94°C, 15 sec), annealing (65°C, 30 sec) and extension (68°C, 1 min) was performed. By this PCR, DNA fragments, wherein EcoR I restriction enzyme recognition sites were added to 5' end and 3' end of the DNA sequences encoding hemoglobin α-chain and β-chain, respectively, were obtained. The DNA fragments amplified by PCR were purified by phenol extraction, ethanol precipitation, then digested with restriction enzyme EcoR I (manufactured by Takara Shuzo Co., Ltd.). After the restriction enzyme treatment, DNA fragments were subjected to 2% agarose electrophoresis, and bands corresponding to each of DNA fragments (DNA fragment encoding hemoglobin α-chain;Hbα, SEQ ID NO: 13 and DNA fragment encoding hemoglobin β-chain; Hbβ, SEQ ID NO: 14) were cut out, and subjected to gel extraction (QIAquick Gel Extraction Kit, manufactured by QIAGEN).

(2) ligation of hemoglobin gene

[0057] E. coli JM109 (manufactured by Takara Shuzo Co., Ltd.) was transfected with pA0815 (manufactured by Invitrogen), a shuttle vector for Escherichia coli and yeast, to perform transformation. The transformant, which was transfected with pA0815, was screened in ampicillin-added medium, and the plasmid was purified from the obtained transformant (QIAprep Spin Miniprep Kit, manufactured by QIAGEN). The plasmid was digested with EcoR I (manufactured by Takara Shuzo Co., Ltd.), double-digested with EcoR I and Sal I (manufactured by Takara Shuzo Co., Ltd.) and double-digested with BamH I and Bgl II (manufactured by Takara Shuzo Co., Ltd.), by which restriction enzyme map was constructed, and the plasmid was confirmed to be an objective plasmid vector.

[0058] Escherichia coli, wherein the transfection of pA0815 was confirmed, was further cultured, and the plasmid was

purified from grown bacterial bodies (QIAGEN plasmid Maxi Kit, manufactured by QIAGEN). Purified pA0815 was digested with EcoR I (manufactured by Takara Shuzo Co., Ltd.), purified by phenol extraction and ethanol precipitation, after which dephosphorylation treatment was performed (Alkaline Phosphatase E. coli C75, manufactured by Takara Shuzo Co., Ltd.).

[0059] Each of Hbα and Hbβ was ligated to dephosphorylation-treated pA0815 (DNA Ligation kit Ver. 1, manufactured by Takara Shuzo Co., Ltd.) to give Hbα-pAO815 and Hbβ-pAO815, respectively. Each of the obtained Hbα-pAO815 and Hbβ-pAO815 was transfected into E. coli JM109 (manufactured by Takara Shuzo Co., Ltd.) to perform transformation. The transformants, which were transfected with either the objective plasmids Hbα-pA0815 or Hbβ-pAO815, were screened in ampicillin-added medium, and the plasmids were purified from the obtained transformants (QIAprep Spin Miniprep Kit, QIAGEN manufactured by). The plasmids were digested with EcoR I (manufactured by Takara Shuzo Co., Ltd.), and double-digested with BamH I and Bgl II (manufactured by Takara Shuzo Co., Ltd.), by which restriction enzyme map was constructed, and the plasmids were confirmed to be Hbα-pAO815 and Hbβ-pAO815.

[0060] Furthermore, full sequences of hemoglobin α-chain and β-chain gene were confirmed by ABI Prism 310 Genetic Analyzer (Applied Biosystems) using 5' AOX1 sequencing primer of SEQ ID NO: 15 (manufactured by Invitrogen), Hbα-sequence primer 1 of SEQ ID NO: 16 and Hbα-sequence primer 2 of SEQ ID NO: 17 for α-chain, and 5' AOX1 sequencing primer of SEQ ID NO: 15 (manufactured by Invitrogen), Hbβ-sequence primer 1 of SEQ ID NO: 18 and Hbβ-sequence primer 2 of SEQ ID NO: 19 for β-chain.

[0061] Escherichia coli, wherein the transfection of Hbα-pAO815 or Hbβ-pAO815 were confirmed, was further cultured, and the plasmids were purified from grown bacterial bodies (QIAGEN plasmid Maxi Kit, manufactured by QIAGEN). Purified Hbα-pAO815 was digested with BamH I and Bgl II (manufactured by Takara Shuzo Co., Ltd.), after which a band corresponding to Hbα expression cassette was cut out, and gel extraction was performed (QIAquick Gel Extraction Kit, manufactured by QIAGEN).

[0062] On the other hand, purified Hbβ-pAO815 was digested with BamH I (manufactured by Takara Shuzo Co., Ltd.) without performing dephosphorylation treatment, and purified by phenol extraction and ethanol precipitation.

[0063] Hbα expression cassette was ligated to Hbβ-pAO815 (DNA Ligation kit Ver. 1, manufactured by Takara Shuzo Co., Ltd.), which was enzyme-digested by BamH I, to give Hbβ-Hbα-pAO815. Obtained Hbβ-Hbα-pAO815 was transfected into E. coli DH5α (manufactured by Toyobo Co., Ltd.) to perform transformation. The transformant, which was transfected with the objective plasmid Hbβ-Hbα-pAO815, was screened in ampicillin-added medium, and the plasmid was purified from the obtained transformant (QIAprep Spin Miniprep Kit, manufactured by QIAGEN). The plasmid was digested with EcoR I (manufactured by Takara Shuzo Co., Ltd.), and double-digested with BamH I and Bgl II (manufactured by Takara Shuzo Co., Ltd.), by which restriction enzyme map was constructed, and the plasmid was confirmed to be Hbβ-Hbα-pAO815. Fig. 2 shows the procedure for construction of Hbβ-Hbα-pAO815.

(3) expression of hemoglobin

[0064] Hbβ-Hbα-pAO815 was digested with restriction enzyme Sal I, purified by phenol extraction and ethanol precipitation, and subsequently transformed into HIS4 gene locus of Pichia yeast (GS115 strain) by homologous recombination using an electroporation apparatus (Gene Pulser II Electroporation System, manufactured by BIO-RAD) (see Fig. 3). The obtained transformant was cultured in BMMY liquid medium, and stocked in glycerol after confirmation of expression of hemoglobin.

(4) purification of hemoglobin

[0065] The transformed Pichia yeast was cultured in BMGY liquid medium for 48 hr, and subsequently in BMMY medium for 96 hr as adding 1% methanol every 12 hr. The yeast was precipitated by centrifugation (6,000 g x 10 min), resuspended in disrupt buffer, and glass beads (425 - 600 μm) was added, after which the suspension was stirred intensively to disrupt the yeast.

[0066] The disrupted fungus body was separated by centrifugation at 10,000 g for 30 min, and the supernatant was collected. This was dialyzed against 10 mM Tris-HCl buffer (pH 7.5), and passed through Q-Sepharose™ Fast Flow column (manufactured by Amersham Biosciences), furthermore, bonded to the Q-Sepharose™ Fast Flow column by pH adjustment to 8.5, and hemoglobin was eluted by concentration gradient of 0 to 300 mM NaCl. This was replaced with 200 mM sodium acetate buffer (pH 5.5), bonded to Blue Sepharose CL-6B column (manufactured by Amersham Biosciences), and hemoglobin was eluted by concentration gradient of 0 to 3 M NaCl. Subsequently, this eluate was dialyzed against 0.5 M ammonium sulfate/100 mM sodium phosphate buffer (pH 7.0), bonded to HiTrap Butyl FF column (manufactured by Amersham Biosciences), and hemoglobin was eluted with 70% ethanol. Finally, CO was blown into the eluate and the eluate was preserved at 4°C.

(5) confirmation of hemoglobin

[0067] The final sample was subjected to SDS polyacrylamide gel electrophoresis, and a single band of predicted molecular weight, about 15,000, was detected. Also, N-terminal amino acid analysis was performed, and it showed that the band completely corresponded with the amino acid sequence of human blood-derived hemoglobin. Furthermore, molecular weights of α-chain and β-chain were estimated by MALDI TOF-MS, 15,122 and 15,866, respectively, corresponding with calculated weights.

[0068] The production method of the present invention is free of a risk of contamination with virus and the like. Therefore, it is extremely useful in that it can conveniently provide human hemoglobin in large amounts, which can be safely administered to the living body for medical purposes.

[0069] While the present invention has been described with emphasis on preferred embodiments, it is obvious to those skilled in the art that the preferred embodiments can be modified. The present invention intends that the present invention can be embodied by methods other than those described in detail in the present specification. Accordingly, the present invention encompasses all modifications encompassed in the gist and scope of the appended "CLAIMS."

[0070] In addition, the contents disclosed in any publication cited herein, including patents and patent applications, are hereby incorporated in their entireties by reference, to the extent that they have been disclosed herein.

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Claims

1. A production method of a functional recombinant human hemoglobin, which comprises transforming a yeast of the genus Pichia with an expression vector containing a DNA encoding a human hemoglobin α-chain, which is under regulation of a promoter functional in a yeast of the genus Pichia, and an expression vector containing a DNA encoding a human hemoglobin β-chain, which is under regulation of a promoter functional in a yeast of the genus Pichia, culturing the yeast in a medium, and recovering the human hemoglobin from the obtained culture.

2. The method of claim 1, wherein the DNA encoding the human hemoglobin α-chain and the DNA encoding the human hemoglobin β-chain are on the same vector.

3. The method of claim 2, wherein the DNA encoding the human hemoglobin β-chain is at the upstream side in the transcriptional direction.

4. The method of any of claims 1 to 3, wherein the DNA encoding the human hemoglobin α-chain and the DNA encoding the human hemoglobin β-chain are under regulation of different promoters.

5. The method of any of claims 1 to 4, wherein the DNA encoding the human hemoglobin α-chain and the DNA encoding the human hemoglobin β-chain are under regulation of an alcoholoxidase 1 promoter.

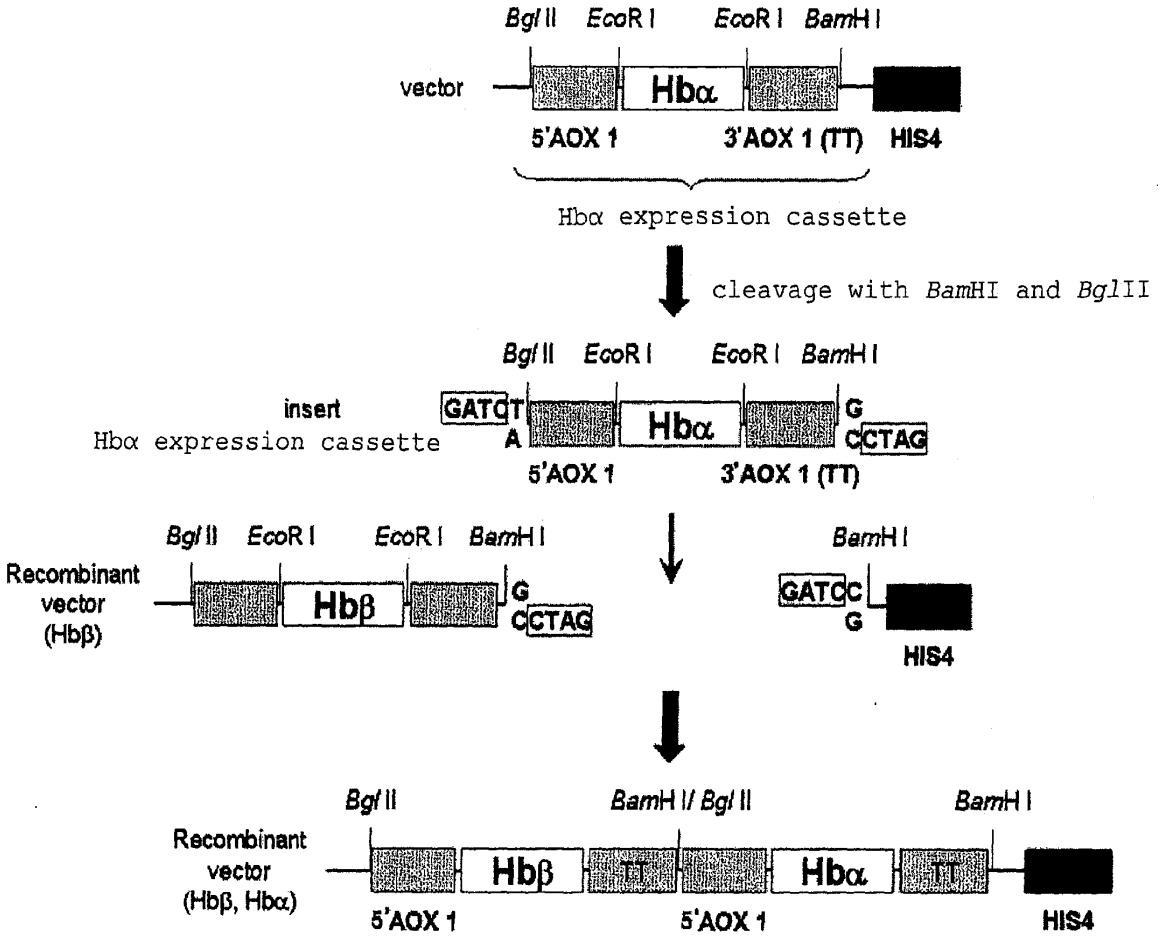
6. The method of claim 5, wherein the transformed yeast of the genus Pichia is cultivated in a liquid medium requiring methanol as a single carbon source.

7. The method of claim 6, wherein the culture is a feeding culture.

8. The method of any of claims 1 to 7, wherein the yeast of the genus Pichia is Pichia pastoris.

9. An expression vector comprising an alcoholoxidase 1 promoter, a DNA encoding the human hemoglobin β-chain, a terminator functional in a yeast of the genus Pichia, an alcoholoxidase 1 promoter, a DNA encoding a human hemoglobin α-chain and a terminator functional in a yeast of the genus Pichia in this order from the upstream side in the transcriptional direction.

FIG. 1



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FIG. 2

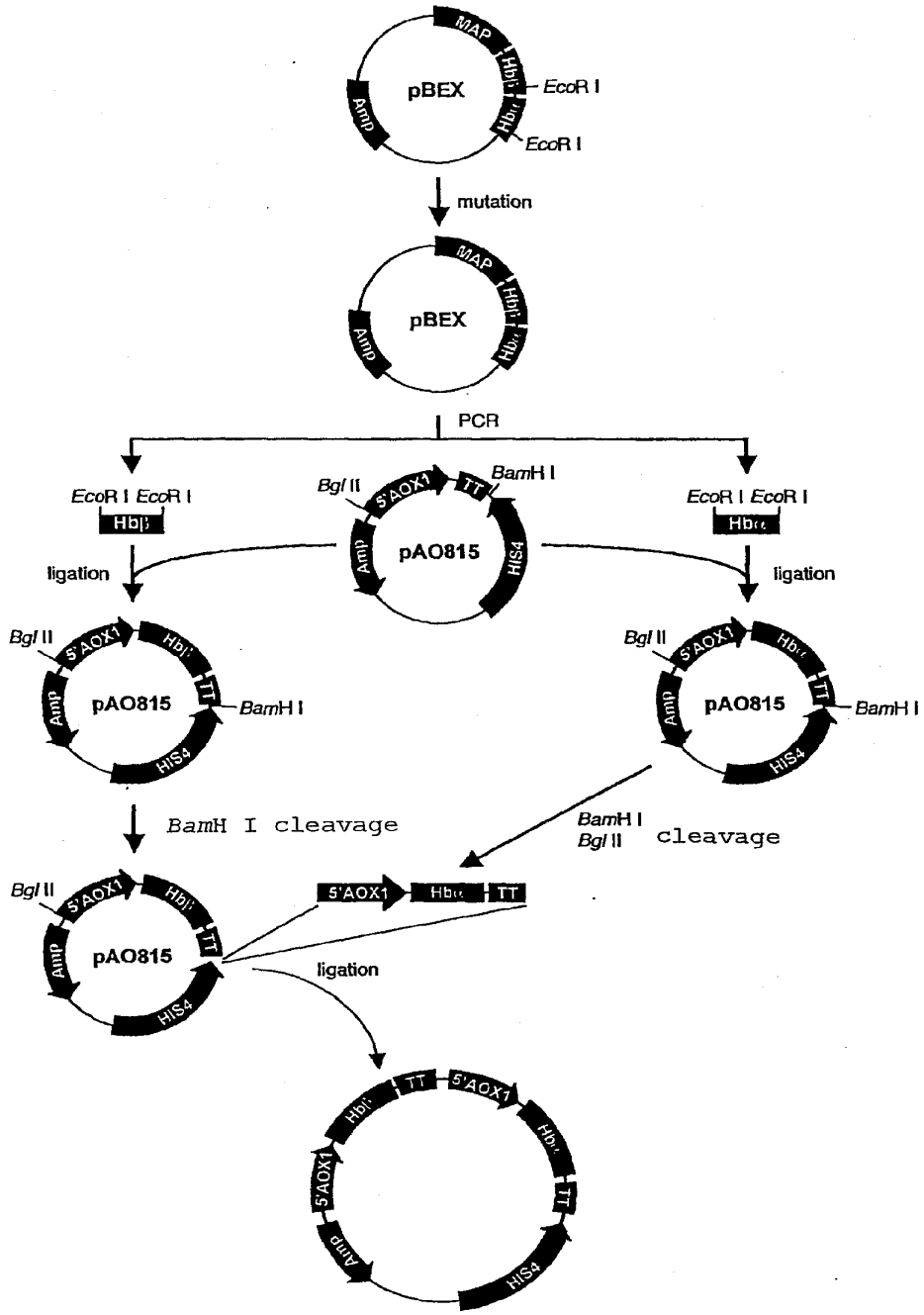
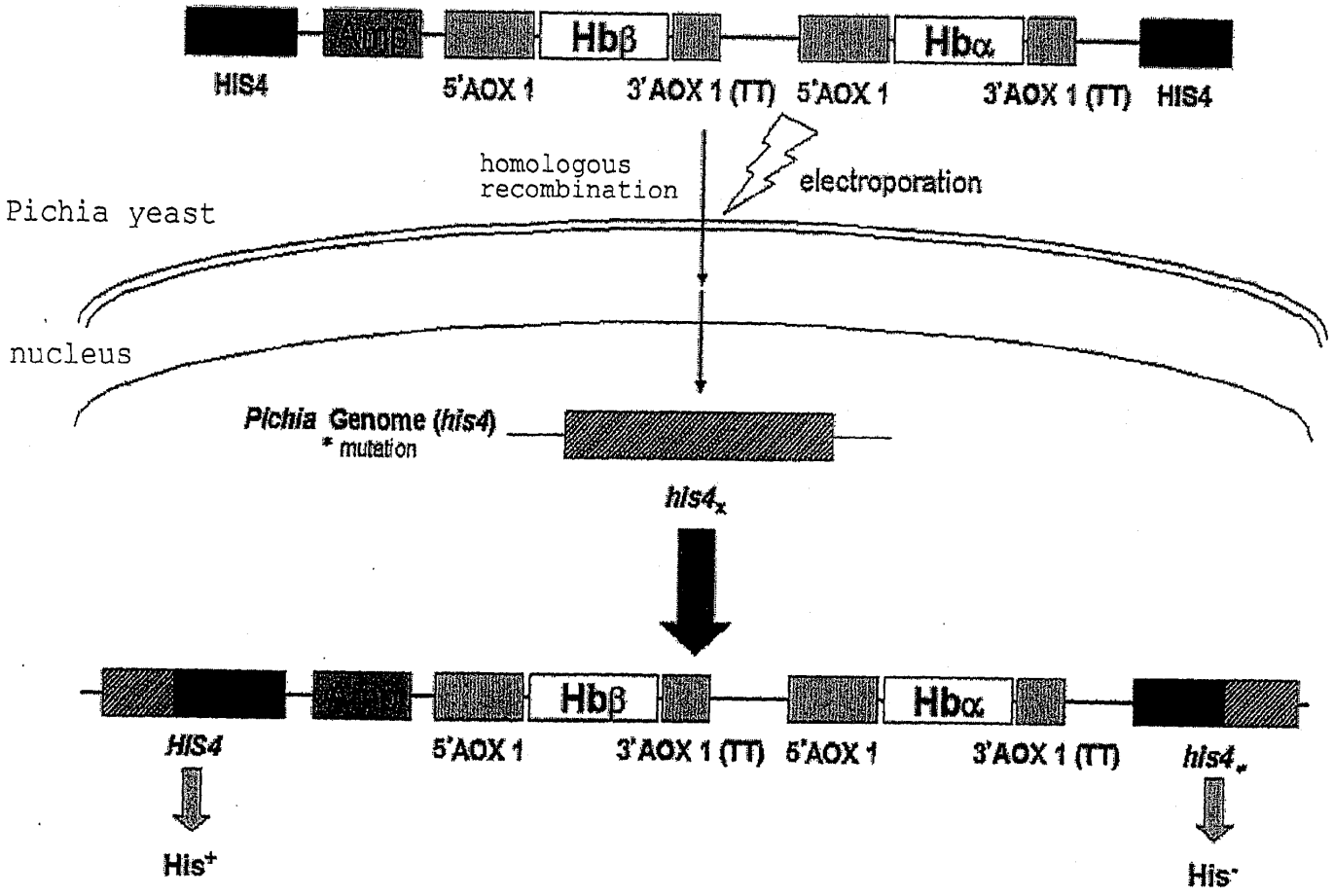

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EcoR I
mutation
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ligation
EcoR I EcoR I
Hbα
ligation
Bgl II
5'AOX1
Hbβ
pAO815
TT
BamH I
Amp
HIS4
Bgl II
5'AOX1
Hbα
pAO815
TT
BamH I
Amp
HIS4
BamH I cleavage
BamH I
Bgl II
cleavage
Bgl II
5'AOX1
Hbβ
pAO815
TT
Amp
HIS4
5'AOX1
Hbα
TT
ligation
TT
5'AOX1
Hbβ
5'AOX1
Hbα
TT
Amp
HIS4

FIG. 3



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EUROPEAN SEARCH REPORT

Application Number
EP 07 12 0389

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	2 April 2008	Loubradou, Gabriel

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
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L : document cited for other reasons

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
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国 际 局

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(81) 指定国 (除另有指明，要求每一种可提供的国家保护): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW。

(84) 指定国 (除另有指明，要求每一种可提供的地区保护): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), 欧亚 (AM, AZ, BY, KG, KZ, RU, TJ, TM), 欧洲 (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)。

本国际公布:
— 包括国际检索报告(条约第 21 条(3))。
— 包括说明书序列表部分(细则 5.2(a))。

WO 2014/008729 A1

(54) Title: METHOD OF ELIMINATING DEPENDENCE OF METHANOL INDUCED PROMOTER ON SINGLE METHANOL CARBON SOURCE

(54) 发明名称 : 消除甲醇诱导型启动子单一甲醇碳源依赖性的方法

(57) Abstract: Provided is a method of eliminating the dependence of methanol induced promoter on a single methanol carbon source for expressing foreign polypeptide. The method comprises activating the expression of the promoter requiring methanol induction by increasing the expression quantity of Mit1 polypeptide in cells of methylotrophic yeast, such that the promoter originally depending on methanol induction no longer depends on single methanol and can also express foreign polypeptide.

(57) 摘要: 提供了一种消除甲醇诱导型启动子单一甲醇碳源依赖性而表达外源多肽的方法。该方法通过在甲醇营养型酵母细胞内增加 Mit1 多肽的表达量，激活需要甲醇诱导的启动子的表达，使得原本依赖甲醇诱导的启动子不再依赖单一性甲醇也可以表达外源多肽。

消除甲醇诱导型启动子单一甲醇碳源依赖性的方法

技术领域

本发明属于生物工程领域；更具体地，本发明涉及一种消除甲醇诱导型启动子单一甲醇碳源依赖性而表达外源多肽的方法。

背景技术

甲醇营养型酵母(包括 *Pichia*，*Hansenula*，*Candida*，*Torulopsis* 等)表达系统由于其高效的外源多肽表达能力已经在工业生产、制药等领域被非常广泛的运用。其特点在于，它们拥有能够被甲醇高效诱导的启动子(*AOX1* 启动子、*DHAS* 启动子、*FDH* 启动子、*MOX* 启动子、*AOX2* 启动子、*ZZA1*、*PEX5*-、*PEX8*-、*PEX14*-启动子等)，并且这些启动子严格依赖甲醇，其它碳源(如葡萄糖，甘油等)会抑制这些启动子的表达。

随着石油危机的爆发，利用甲醇生产单细胞蛋白，成本变高。之所以利用甲基营养型母酵母表达系统表达如此之多的外源多肽，是因为其具有其他表达系统不可比拟的优势：基因操作简单，外源多肽表达量高，既可以胞内表达，也可以分泌表达；外源多肽基因遗传稳定；作为真核表达系统，具有真核生物的亚细胞结构，具有糖基化、脂肪酰化、蛋白磷酸化等翻译后修饰加工功能；

由于被利用的范围越来越广，在实际的发酵放大过程中遇到很多问题：(1) 表达所用的启动子需要甲醇的诱导，甲醇有毒易燃，在大规模工业发酵中需要进行特别的防暴设计；(2) 甲醇发酵强耗氧，一般靠增大空气的通气量和提高转速很难满足氧气的需求而需要通纯氧，甲醇代谢需要氧气的量是葡萄糖为碳源所需氧气量的三至四倍。消耗的甲醇越多需要的纯氧越多，这给实际的工业化生产带来很大的麻烦。另外消耗的甲醇越多所产的热量也越大，所需设备的冷却能力要求越高；(3) 甲醇作为一种石油化学产品，不适合于一些食品领域添加剂的生产；(4) 甲醇代谢会产生 H_2O_2，会导致所表达多肽的水解。

因此，如果得到一种能够利用非甲醇诱导甲醇诱导型启动子表达的方法，使原本依赖甲醇诱导的启动子的高效转录不依赖于甲醇，而可用其他碳源来诱导这些启动子的表达，这对于实现工业上利用非甲醇高效表达外源多肽具有积极意义。

发明内容

本发明的目的在于提供一种消除甲醇诱导型启动子单一甲醇碳源依赖性而驱

动甲醇诱导型启动子表达外源多肽的方法。

本发明的另一目的还在于提供重组的甲醇营养型酵母，其可以利用消除甲醇诱导型启动子单一甲醇碳源依赖性来表达外源多肽。

在本发明的第一方面，提供一种消除甲醇诱导型启动子单一甲醇碳源依赖性而驱动外源多肽编码基因表达的方法，包括：

(1) 提供甲醇营养型酵母，所述甲醇营养型酵母含有：

表达盒 1，其表达外源的 Mit1 多肽；以及

表达盒 2，包括甲醇诱导型启动子及与之操作性连接的外源多肽编码基因；

(2) 在无甲醇条件或非单一甲醇碳源条件下培养(1)的甲醇营养型酵母。

在一个优选例中，所述的 Mit1 多肽是转录激活相关因子，使得原本依赖甲醇诱导的启动子不再依赖甲醇也可表达外源多肽。

在另一优选例中，所述的 Mit1 多肽的功能为：激活甲醇代谢中需要甲醇诱导的启动子表达。

在另一优选例中，所述的 Mit1 是 PpMit1。

在另一优选例中，所述表达盒 2 在甲醇营养型酵母中以单拷贝或多拷贝存在，例如 1-20 拷贝，如 15，10，8，6，5，3，2 拷贝。

在另一优选例中，所述的甲醇诱导型启动子包括(但不限于)：*AOX1* 启动子、*DHAS* 启动子、*DAS* 启动子、*FDH* 启动子、*FMDH* 启动子、*MOX* 启动子、*AOX2* 启动子、*ZZA1*、*PEX5*-、*PEX8*-、*PEX14*-启动子、*PMP20* 启动子、*PMP47* 启动子、*AOD1* 启动子、*AOD2* 启动子。

在另一优选例中，所述的甲醇营养型酵母包括 (但不限于)：毕赤酵母(*Pichia*)，汉逊酵母(*Hansenula*)，假丝酵母(*Candida*)，球拟酵母(*Torulopsis*)；较佳地，所述的毕赤酵母(*Pichia*)包括(但不限于)：GS115，*MIG1* 基因和 *MIG2* 基因不表达的毕赤酵母(Δ*mig1*Δ*mig2*)，*NRG1* 基因、*MIG1* 基因和 *MIG2* 基因不表达的毕赤酵母(Δ*mig1*Δ*mig2*Δ*nrg1*)，或 *HXS1* 基因不表达的毕赤酵母菌株。

在另一优选例中，述的 Mit1 多肽选自下组：

(a) SEQ ID NO: 2 所示氨基酸序列的多肽；或

(b) 将 SEQ ID NO: 2 所示氨基酸序列经过一个或多个(如 1-30 个，更佳地 1-20 个，更佳地 1-15 个，更佳地 1-10 个，更佳地 1-5 个或 1-3 个)氨基酸残基的取代、缺失或添加而形成的，且具有利用非甲醇碳源诱导甲醇诱导型启动子表达外源多肽功能的由(a)衍生的多肽；

(c) 与(a)限定的序列在具有 70%以上(较佳地 80%以上，更佳地 90%以上，更佳地 95%以上，更佳地 98%以上，如 99%或更高)的序列相同性的，且具有利用非甲醇碳源诱导甲醇诱导型启动子表达外源多肽功能的由(a)衍生的多肽。

在另一优选例中，所述的 Mit1 多肽的编码基因的 NCBI Reference Sequence 为 XM_002493021.1。

在另一优选例中，所述的 Mit1 多肽的编码基因具有 SEQ ID NO: 1 所示的核苷酸序列。

在另一优选例中，所述的 Mit1 多肽的编码基因在甲醇存在的情况下诱导表达，在甘油、葡萄糖等碳源存在的情况下不表达。

在另一优选例中，所述的表达盒 1 中，包括启动子以及与之操作性连接的 Mit1 多肽的编码基因；较佳地，该启动子包括(但不限于)：组成型启动子，诱导型启动子，组织或器官特异性启动子，时空特异性表达启动子。

在另一优选例中，表达盒 1 中，所述启动子包括(但不限于)：*GAP* 启动子、*PGK1* 启动子、*MIT1* 启动子等任意能使 *MIT1* 过表达的启动子。

在另一优选例中，所述的表达盒 1 在甲醇营养型酵母中以单拷贝或多拷贝存在，例如 1-20 拷贝，如 15，10，8，6，5，3，2 拷贝。

在另一优选例中，步骤(2)中，采用存在甘油和/或葡萄糖的酵母培养基。

在本发明的另一方面，提供 Mit1 多肽或其编码基因的用途，用于消除甲醇诱导型启动子单一甲醇碳源依赖性而驱动外源多肽编码基因表达。

在本发明的另一方面，提供一种重组的甲醇营养型酵母，所述甲醇营养型酵母含有：

表达盒 1，其能够表达外源的 Mit1 多肽；以及

表达盒 2，包括甲醇诱导型启动子及与之操作性连接的外源多肽编码基因。

在一个优选例中，所述的甲醇营养型酵母包括 (但不限于)：毕赤酵母(*Pichia*)，汉逊酵母(*Hansenula*)，假丝酵母(*Candida*)，球拟酵母(*Torulopsis*)；较佳地，所述的毕赤酵母(*Pichia*)包括(但不限于)：GS115，*MIG1* 基因和 *MIG2* 基因不表达的毕赤酵母(Δ*mig1*Δ*mig2*)，*NRG1* 基因、*MIG1* 基因和 *MIG2* 基因不表达的毕赤酵母(Δ*mig1*Δ*mig2*Δ*nrg1*)，或 *HXS1* 基因不表达的毕赤酵母菌株。

在一个优选例中，所述的所述的 *MIG1* 基因的核苷酸序列如 SEQ ID NO: 9 所示；

所述的 *MIG2* 基因的核苷酸序列如 SEQ ID NO: 10 所示；或

所述的 *HXS1* 基因的核苷酸序列如 SEQ ID NO: 3 所示。

所述的 *NRG1* 基因的核苷酸序列如 SEQ ID NO:8 所示(NCBI Reference Sequence: XM_002493138.1)。

本发明的其它方面由于本文的公开内容，对本领域的技术人员而言是显而易见的。

附图说明

图 1、pGAPZαA 的质粒图谱示意图。

图 2、载体 pAG32 的质粒图谱示意图。

图 3、野生型及 GS115-MIT1 分别在非甲醇或含甲醇(甘油+甲醇)的培养基中培养，并进行 Aox 显色反应的结果。

图 4、野生型及 GS115-MIT1 分别在非甲醇或含甲醇的培养基中培养，并进行 Aox 酶活测定的结果。

图 5、菌株 GS115-MIT1-GFP 在 YND 液体培养基中过夜预培养，然后分别转移至非甲醇或含甲醇的培养基中培养，取样后用流式细胞仪检测样品中 GFP 的几何平均荧光强度。

图 6、野生型及 Δ*mig1*Δ*mig2*-MIT1 和 Δ*hxs1*-MIT1 菌株分别在非甲醇或含甲醇的培养基中培养，生长到对数生长期后取样 1ml，在离心后的菌体中加入 Aox 酶活显色液显色。

图 7、野生型及 Δ*mig1*Δ*mig2*-MIT1 和 Δ*hxs1*-MIT1 菌株分别在非甲醇或含甲醇的培养基中培养，生长到对数生长期后取样提取总蛋白，经 Bradford 法蛋白定量后进行 Aox 酶活的测定。

图 8、野生型及 Δ*mig1*Δ*mig2*-MIT1 和 Δ*hxs1*-MIT1 菌株分别在非甲醇或含甲醇的培养基中培养，取样后用流式细胞仪检测样品中 GFP 的几何平均荧光强度。

图 9、载体 pPIC3.5K 的质粒图谱示意图。

图 10、载体 pUC18 的质粒图谱示意图

图 11、野生型及 Δ*mig1*Δ*mig2*Δ*nrg1*-MIT1 菌株分别在非甲醇或含甲醇的培养基中培养，生长到对数生长期后取样提取总蛋白，经 Bradford 法蛋白定量后进行 Aox 酶活的测定。

图 12、野生型及 Δ*mig1*Δ*mig2*Δ*nrg1*-MIT1 菌株分别在非甲醇或含甲醇的培养基中培养，取样后用流式细胞仪检测样品中 GFP 的几何平均荧光强度。

具体实施方式

为了克服目前甲醇诱导型启动子在发酵时对于甲醇诱导的依赖性，本发明人经过深入的研究，揭示了一种消除甲醇诱导型启动子单一甲醇碳源依赖性的方法，通过在甲醇营养型酵母细胞内增加 Mit1(甲醇诱导的转录因子 1；Methanol Induced Transcription Factor 1)多肽的表达量，激活甲醇代谢中需要单一甲醇诱导的启动子表达，以此使得原本依赖甲醇诱导的启动子不再依赖甲醇也可表达外源多肽。

术语

如本文所用，所述的“启动子”是指一种核酸序列，其通常存在于目的基因编码序列的上游(5’端)，能够引导核酸序列转录为 mRNA。一般地，启动子或启动子区提供 RNA 聚合酶和正确起始转录所必需的其它因子的识别位点。在本文中，所述的启动子或启动子区包括启动子的活性变异体，该变异体可以是天然发生的等位变异体或非天然发生的变异体。所述变异体包括取代变异体、缺失变异体和插入变异体。

如本文所用，所述的“甲醇诱导型启动子”是与甲醇代谢有关的酶的启动子。在现有技术中，这些启动子通过向生长培养基中添加甲醇，可以控制外源多肽由所述启动子的表达。所述的“甲醇诱导型启动子”可以由本领域技术人员使用常规技术从酵母中分离获得。

如本文所用，所述的“单一甲醇(碳源)诱导”是指启动子需要以甲醇为唯一碳源进行诱导来驱动与之操作性连接的基因的表达，在非甲醇为唯一碳源(如甲醇+葡萄糖)的条件下不能驱动与之操作性连接的基因的表达。所述的“消除单一甲醇诱导”是指使得启动子在不以甲醇为唯一碳源(如甲醇+葡萄糖，或葡萄糖，或甘油)的条件下就可驱动与之操作性连接的基因的表达。“非单一甲醇(碳源)诱导”是指除了甲醇碳源外，还存在至少一种非甲醇的碳源。

如本文所用，所述的“组成型启动子”是指在其调控下不同组织器官和发育阶段的基因表达没有明显差异的一类启动子。

如本文所用，所述的“诱导型启动子”可根据需要在特定细胞生长阶段或特定生长环境下，快速诱导基因转录的“开”与“关”或者“高”与“低”。根据来源，可将诱导型启动子分为天然存在的启动子和人工构建的启动子。

如本文所用，所述的“组织或器官特异性启动子”是指基因转录一般只发生在某些特定器官或组织中的启动子。

如本文所用，“外源的”或“异源的”是指来自不同来源的两条或多条核酸或蛋白质序列之间的关系。例如，如果启动子与目的基因序列的组合通常不是天然存在的，则启动子对于该目的基因来说是外源的。特定序列对于其所插入的细胞或生物体来说是“外源的”。

如本文所用，所述的“表达盒”是指包含有表达目的多肽(本发明中为外源多肽或 Mit1 多肽)所需的所有必要元件的基因表达系统，通常其包括一下元件：启动子、编码多肽的基因序列，终止子；此外还可选择性包括信号肽编码序列等。这些元件是操作性相连的。

如本文所用，所述的“甲醇营养型酵母”是指可利用甲醇作为唯一碳源的酵母。包括来自汉逊酵母属(*Hansenula*)，毕赤酵母属(*Pichia*)，球拟酵母属(*Torulopsis*)，假丝酵母属(*Candida*)等的酵母。

如本文所用，所述的“可操作性连接”是指两个或多个核酸区域或核酸序列的功能性的空间排列。例如：启动子区被置于相对于目的基因核酸序列的特定位置，使得核酸序列的转录受到该启动子区域的引导，从而，启动子区域被“可操作地连接”到该核酸序列上。

如本文所用，术语“严格条件”是指：(1)在较低离子强度和较高温度下的杂交和洗脱，如 0.2×SSC，0.1%SDS，60℃；或(2)杂交时加有变性剂，如 50%(v/v)甲酰胺，0.1%小牛血清/0.1%Ficoll，42℃等；或(3)仅在两条序列之间的相同性至少在 50%，优选 55%以上、60%以上、65%以上、70%以上、75%以上、80%以上、85%以上或 90%以上，更优选是 95%以上时才发生杂交。

如本文所用，所述的“含有”，“具有”或“包括”包括了“包含”、“主要由……构成”、“基本上由……构成”、和“由……构成”；“主要由……构成”、“基本上由……构成”和“由……构成”属于“含有”、“具有”或“包括”的下位概念。

Mit1 多肽

本发明揭示了 Mit1 多肽在消除甲醇诱导型启动子单一甲醇碳源依赖性而驱动外源多肽编码基因表达中的应用。在对依赖甲醇控制外源多肽表达的启动子(甲醇诱导型启动子)的研究工作中，本发明人意外发现 Mit1 多肽可以解除甲醇诱导

型启动子对于甲醇的依赖性，使其可利用非单一甲醇碳源或非甲醇碳源进行外源多肽的表达。

本发明还包括所述的 Mit1 多肽的片段、衍生物和类似物。如本文所用，术语“片段”、“衍生物”和“类似物”是指基本上保持本发明的 MIT1 多肽相同的生物学功能或活性的多肽。本发明的多肽片段、衍生物或类似物可以是(i)有一个或多个保守或非保守性氨基酸残基(优选保守性氨基酸残基)被取代的多肽，而这样的取代的氨基酸残基可以是也可以不是由遗传密码编码的，或(ii)在一个或多个氨基酸残基中具有取代基团的多肽，或(iii)成熟多肽与另一个化合物(比如延长多肽半衰期的化合物，例如聚乙二醇)融合所形成的多肽，或(iv)附加的氨基酸序列融合到此多肽序列而形成的多肽(如前导序列或分泌序列或用来纯化此多肽的序列或多肽原序列，或融合多肽)。根据本文的定义这些片段、衍生物和类似物属于本领域熟练技术人员公知的范围。

在本发明中，术语“Mit1 多肽”指具有利用非甲醇碳源诱导甲醇诱导型启动子表达外源多肽功能的 SEQ ID NO: 2 序列的多肽。该术语还包括具有利用非甲醇碳源诱导甲醇诱导型启动子表达外源多肽功能的、SEQ ID NO: 2 序列的变异形式。这些变异形式包括(但并不限于)：若干个(通常为 1-50 个，较佳地 1-30 个，更佳地 1-20 个，最佳地 1-10 个，还更佳如 1-8 个或 1-5 个)氨基酸的缺失、插入和/或取代，以及在 C 末端和/或 N 末端添加或缺失一个或数个(通常为 20 个以内，较佳地为 10 个以内，更佳地为 5 个以内)氨基酸。例如，在本领域中，用性能相近或相似的氨基酸进行取代时，通常不会改变蛋白质的功能。又比如，在 C 末端和/或 N 末端添加或减少一个或数个氨基酸通常也不会改变蛋白质的功能。

多肽的变异形式包括：同源序列、保守性变异体、等位变异体、天然突变体、诱导突变体、在高或低的严紧度条件下能与编码 Mit1 多肽的 DNA 杂交的 DNA 所编码的多肽、以及利用抗 Mit1 多肽的抗血清获得的多肽或蛋白。本发明还提供了其他多肽，如包含 Mit1 多肽或其片段的融合多肽。

本发明还提供 Mit1 多肽或多肽的类似物。这些类似物与天然 Mit1 多肽的差别可以是氨基酸序列上的差异，也可以是不影响序列的修饰形式上的差异，或者兼而有之。这些多肽包括天然或诱导的遗传变异体。诱导变异体可以通过各种技术得到，如通过辐射或暴露于诱变剂而产生随机诱变，还可通过定点诱变法或其他已知分子生物学的技术。类似物还包括具有不同于天然 L-氨基酸的残基(如 D-氨基酸)的类似物，以及具有非天然存在的或合成的氨基酸(如 β、γ-氨基酸)的类

似物。应理解，本发明的多肽并不限于上述例举的代表性的多肽。

本发明还提供了编码本发明 Mit1 多肽或其保守性变异多肽的多核苷酸序列。

本发明的多核苷酸可以是 DNA 形式或 RNA 形式。DNA 形式包括 cDNA、基因组 DNA 或人工合成的 DNA。DNA 可以是单链的或是双链的。DNA 可以是编码链或非编码链。编码成熟多肽的编码区序列可以与 SEQ ID NO: 1 序列相同或者是简并的变异体。如本文所用，“简并的变异体”在本发明中是指编码具有 SEQ ID NO: 2 序列或其变异形式的蛋白，但与 SEQ ID NO: 1 中的编码区序列有差别的核酸序列。

本发明还涉及上述多核苷酸的变异体，其编码与本发明有相同的氨基酸序列的多肽或多肽的片段、类似物和衍生物。此多核苷酸的变异体可以是天然发生的等位变异体或非天然发生的变异体。这些核苷酸变异体包括取代变异体、缺失变异体和插入变异体。如本领域所知的，等位变异体是一个多核苷酸的替换形式，它可能是一个或多个核苷酸的取代、缺失或插入，但不会从实质上改变其编码的多肽的功能。

本发明还涉及与上述多核苷酸同源的多核苷酸，它们是与上述多核苷酸具有至少50%，较佳地至少60%，更佳地至少70%，更佳地至少80%相，更佳地至少90%；更佳地至少95%；更佳地至少98%或99%相同性的多核苷酸。这些多核苷酸所编码的蛋白也具有与前述多核苷酸所编码的多肽相同的利用非甲醇碳源诱导甲醇诱导型启动子表达外源多肽的功能。

本发明还涉及与上述的序列杂交且两个序列之间具有至少 50%，较佳地至少 70%，更佳地至少 80%相同性的多核苷酸。本发明特别涉及在严格条件下与本发明所述多核苷酸可杂交的多核苷酸。

本发明的 Mit1 多肽核苷酸全长序列或其片段通常可以用 PCR 扩增法、重组法或人工合成的方法获得。对于 PCR 扩增法，可根据本发明所公开的有关核苷酸序列，尤其是开放阅读框序列来设计引物，并用市售的 cDNA 库或按本领域技术人员已知的常规方法所制备的 cDNA 库作为模板，扩增而得有关序列。当序列较长时，可进行两次或多次 PCR 扩增，然后再将各次扩增出的片段按正确次序拼接在一起。

本发明中，编码 Mit1 多肽的多核苷酸序列可插入到重组表达载体中。术语“重组表达载体”指本领域熟知的细菌质粒、噬菌体、酵母质粒、植物细胞病毒、哺乳动物细胞病毒或其他载体。总之，只要能在宿主体内复制和稳定，任何质粒和

载体都可以用。表达载体的一个重要特征是通常含有复制起点、启动子、标记基因和翻译控制元件。

本领域的技术人员熟知的方法能用于构建含 Mit1 多肽编码 DNA 序列和合适的转录/翻译控制信号的表达载体。这些方法包括体外重组 DNA 技术、DNA 合成技术、体内重组技术等。所述的 DNA 序列可有效连接到表达载体中的适当启动子上，以指导 mRNA 合成。表达载体还包括翻译起始用的核糖体结合位点和转录终止子。

此外，表达载体优选地包含一个或多个选择性标记基因，以提供用于选择转化的宿主细胞的表型性状，如真核细胞培养用的二氢叶酸还原酶、新霉素抗性以及绿色荧光蛋白(GFP)。

包含上述的适当 DNA 序列以及适当启动子或者控制序列的载体，可以用于转化适当的宿主细胞，以使其能够表达蛋白质。

增加 Mit1 多肽表达的方法是本领域周知的。例如，可通过转入携带 Mit1 多肽编码基因的表达构建物使酵母细胞过表达 Mit1 多肽；或可通过用强启动子驱动从而增强 Mit1 多肽的表达；或者通过增强子来增强该 Mit1 多肽的表达。

甲醇诱导型启动子

在现有常规技术中，所述的甲醇诱导型启动子依赖于在培养基中添加甲醇，从而控制外源多肽由所述启动子的表达。而本发明人利用 Mit1 多肽解除了甲醇诱导型启动子对于甲醇的依赖。

本领域技术人员熟悉甲醇依赖型启动子，可使用常规技术从酵母中分离获得。由于甲醇依赖型启动子具有基本相同的工作机制和工作原理，本发明对于甲醇依赖型启动子的种类没有特别的限制。例如，所述的甲醇诱导型启动子包括但不限于：*AOX1* 启动子、*DHAS* 启动子(或者 *DAS* 启动子)、*FDH* 启动子(或者 *FMDH* 启动子)、*MOX* 启动子、*AOX2* 启动子、*ZZA1*、*PEX5*-、*PEX8*-、*PEX14*-启动子、*PMP20* 启动子、*PMP47* 启动子、*AOD1* 启动子、*AOD2* 启动子。

本发明还包括与上述启动子的多核苷酸序列具有至少 70%，更佳地至少 80% (例如 85%、90%、95%、96%、97%、98%、或 99%) 相同性的启动子。这些启动子在引发转录的必要位点及转录起始点的位置上是严格保守的。本发明特别涉及在严格条件下与本发明所述甲醇依赖型启动子的核苷酸序列可杂交的多核苷酸，且该多核苷酸也具有野生型甲醇依赖型启动子的功能。

消除甲醇诱导型启动子单一甲醇碳源依赖性而驱动外源多肽编码基因表达的方法

本发明还提供了一种消除甲醇诱导型启动子单一甲醇碳源依赖性而驱动外源多肽编码基因表达的方法，包括：

(1) 提供甲醇营养型酵母，所述甲醇营养型酵母含有：

表达盒 1，其能够表达外源的 Mit1 多肽；以及表达盒 2，包括甲醇诱导型启动子及与之操作性连接的外源多肽编码基因；

(2) 在无甲醇条件或非单一甲醇碳源条件培养(1)的甲醇营养型酵母。

所述的表达盒 1 和表达盒 2 可以存在于同一表达载体中，也可以存在于不同的表达载体中。

所述表达盒 1 在甲醇营养型酵母中以单拷贝或多拷贝存在，例如 1-20 拷贝，如 15，10，8，6，5，3，2 拷贝。所述的表达盒 2 在甲醇营养型酵母中也可以单拷贝或多拷贝存在，例如 1-20 拷贝，如 15，10，8，6，5，3，2 拷贝。

所述的表达盒2中，包括启动子以及与之操作性连接的Mit1多肽的编码基因。应理解，任何可使得 Mit1 多肽在甲醇营养型酵母中重组表达的启动子都可用于本发明。不限于组成型启动子、诱导型启动子或特异性启动子。本领域技术人员可以根据所需达到的目的进行合适的选择，例如当希望通过一定的条件干预来控制 Mit1 多肽的表达或不表达，则可以选择诱导型启动子。较佳地，表达盒 2 中，所述启动子包括：*GAP* 启动子、*PGK1* 启动子、*MIT1* 启动子等任意能够使得 *MIT1* 过表达的启动子。

步骤(2)中，“无甲醇的条件”是本领域技术人员易于建立的，也即，在常规应用的甲醇营养型酵母培养基中，不加入甲醇作为碳源，取代以其它类型的碳源，常用的碳源是本领域技术人员熟知的，例如但不限于：甘油、葡萄糖、淀粉(含淀粉水解液，木薯淀粉，玉米淀粉，纤维素类水解液等)、蔗糖、麦芽糖等。较佳地，采用以甘油和/或葡萄糖为碳源的酵母培养基。类似地，“非单一甲醇碳源条件”也是本领域技术人员易于建立的。

重组甲醇营养型酵母

基于本发明的新发现，还提供了一种重组的甲醇营养型酵母，所述甲醇营养型酵母含有：表达盒 1，其能够表达外源的 MIT1 多肽；以及表达盒 2，包括甲醇

诱导型启动子及与之操作性连接的外源多肽编码基因。附加条件是，所述的表达盒 1 和表达盒 2 可以存在于同一表达载体中，也可以存在于不同的表达载体中。

任何甲醇营养型酵母均可被应用于本发明中，以构建上述重组的甲醇营养型酵母。例如，所述的甲醇营养型酵母包括但不限于：毕赤酵母(*Pichia*)，汉逊酵母(*Hansenula*)，假丝酵母(*Candida*)，球拟酵母(*Torulopsis*)；较佳地，所述的毕赤酵母(*Pichia*)包括：GS115，*MIG1* 基因和 *MIG2* 基因不表达的毕赤酵母(Δ*mig1*Δ*mig2*)，*NRG1* 基因、*MIG1* 基因和 *MIG2* 基因不表达的毕赤酵母(Δ*mig1*Δ*mig2*Δ*nrg1*)，或基因 *HXS1* 不表达的毕赤酵母菌株。

在本发明的具体实施例中，提供了四株能够利用非甲醇碳源诱导 *AOX1* 启动子表达外源多肽的菌株，GS115-MIT1、Δ*mig1*Δ*mig2*-MIT1、Δ*hxs1*-MIT1 和 Δ*mig1*Δ*mig2*Δ*nrg1*-MIT1。在毕赤酵母野生菌株 GS115、毕赤酵母双缺失菌株 Δ*mig1*Δ*mig2*、毕赤酵母缺失菌株 Δ*hxs1* 和毕赤酵母三缺失菌株 Δ*mig1*Δ*mig2*Δ*nrg1* 中分别过表达了转录激活相关基因 *MIT1*，构建了基因过表达菌株 GS115-MIT1、Δ*mig1*Δ*mig2*-MIT1、Δ*mig1*Δ*mig2*Δ*nrg1*-MIT1 和 Δ*hxs1*-MIT1。经 Aox1 酶活检测发现，甘油可以诱导 GS115-MIT1 的 *AOX1* 启动子的表达，甘油或者葡萄糖均能诱导 Δ*mig1*Δ*mig2*-MIT1、Δ*hxs1*-MIT1 和 Δ*mig1*Δ*mig2*Δ*nrg1*-MIT1 的 *AOX1* 启动子表达。并且用流式细胞仪检测了绿色荧光蛋白 GFP 以 *AOX1* 为启动子时在非甲醇碳源中的诱导表达量。

所述的 Δ*hxs1* 菌株中，与酿酒酵母己糖感应体 Snf3/Rgt2 有很高相似性的一个基因 *HXS1*(GenBank 登录号: 8197942)的编码区缺失。

下面结合具体实施例，进一步阐述本发明。应理解，这些实施例仅用于说明本发明而不用于限制本发明的范围。下列实施例中未注明具体条件的实验方法，通常按照常规条件如 J.萨姆布鲁克等编著，分子克隆实验指南，科学出版社，2002 中所述的条件，或按照制造厂商所建议的条件。除非另外说明，否则百分比和份数按重量计算。

材料

毕赤酵母总蛋白提取技术参照冷泉港公司提供的酵母蛋白提取手册。

所使用的工具酶均购自 TaKaRa 生物公司(大连，中国)，具体的反应条件和使用的方法均参考商品说明书。

下面的商品化质粒和菌株用于基因克隆和蛋白表达：质粒 pGAPZαA、质粒 pPIC3.5k、大肠杆菌 Top10、毕赤酵母菌株 GS115 均购自 Invitrogen 公司。

质粒 pAG32 由质粒 pRDM054 在酶切位点 *Bgl* II 处切除 P_{AOX1}-BFP-SKL 表达体系得到；质粒 pRDM05 获自加州大学圣地亚哥分校。

YPD 培养基：2% 蛋白胨，1% 酵母粉，2% 葡萄糖，2% 琼脂粉；YNB 培养基：0.67% YNB；MGY 培养基：1% 甘油，0.67% YNB； YND 液体培养基：1% 葡萄糖，0.67% YNB。

配制以上培养基时，葡萄糖 115°C 高压灭菌 20 min，甲醇在使用时添加。其它成分 121°C 高压灭菌 20 min。固体培养基加 2% 琼脂粉。

实施例 1、甘油可诱导 *AOX1* 启动子表达的毕赤酵母菌株 GS115-MIT1

1. *PpMIT1* 过表达质粒的构建

采用 PCR，酶切连接的方法，以 pGAPZαA(图 1)为载体在 GAP 启动子下游的 *Asu* II/*Sal* I 两个酶切位点插入 Pp*MIT1* 基因(SEQ ID NO: 1，基因全长 2667bp)，得到的重组质粒称为 pGM 质粒。

以 M1-GAP5 和 M1-AOX1TT 为引物(表 1)，通过 PCR 的方法从 pGM 质粒上扩增得到 pGAP-PpMIT1-AOX1TT 的表达体系(全长 3615 bp)，以 pAG32(图 2)为载体在 *Sac* I/*Spe* I 处插入该表达体系。得到重组质粒为 pGMhph。

表 1. 引物列表

引物	SEQ ID NO:	序列
M1-GAP5	4	5’-CGAGCTCAGATCTTTTTTGTAGAAATGTC-3’
M1-AOX1TT	5	5’- GACTAGTGCACAAACGAAGGTCTC-3’
HXS1-C-5	6	5- CCGGAATTCTAGTCACGGATTGCTTC -3
HXS1-C-3	7	5- ACATGCATGCGTTCCCAATAGATTTCACAA -3

2. 电转毕赤酵母和 GS115-MIT1 菌株的筛选

将 Pp*MIT1* 过表达质粒(pGMhph)电转毕赤酵母菌株 GS115，涂于 4 块加潮霉素的 YPD 固体培养基上，放在 30℃培养箱培养 48-72 小时。将平板上长出的单克隆挑至 10 ml YPD + 潮霉素液体培养基中，30℃摇床培养后，提基因组用 PCR 验证。把 PCR 检验及测序验证后正确的毕赤酵母菌株命名为 GS115-MIT1。

3. Aox 显色反应

将各菌株分别在 0.5% (v/v) 甲醇，1% 甘油，1% 甘油+0.5% (v/v) 甲醇为碳源的 YNB 液体培养基中培养，生长到对数生长期后取样 1 ml，在离心后的菌体中加入 Aox1 酶活显色液显色 (参见 Stasyk O. V., T. Y. Nazarko, and A. A. Sibirny. 2008. Methods of Plate Pexophagy Monitoring and Positive Selection for *ATG* Gene Cloning in Yeasts. *Methods in enzymology*. **451:**229-239。显色底物为 o-dianisidine)。

结果如图 3，可以看到在含有甘油的培养基中生长后，只有菌株 GS115-MIT1 中有 Aox 酶活，野生菌株中均没有酶活。

4. Aox 酶活的测定

将各菌株在 MGY 液体培养基中过夜预培养，然后分别转移到含有 0.5% (v/v) 甲醇，1% 甘油，1% 甘油 + 0.5% (v/v) 甲醇，0.5% (v/v) 甲醇为碳源的 YNB 液体培养基中，培养 10 小时后取样提取总蛋白，经 Bradford 法蛋白定量后进行 Aox1 酶活的测定(方法参见 Verduyn, C., J. P. van Dijken, and W. A. Scheffers. 1984. Colorometric alcohol assays with alcohol oxidase. *J. Microbiol. Methods* **2:**15–25。显色底物为 2,2'-azino-di-(3-ethylbenzthiazoline sulfonate)，ABTS)。

由图 4 可知，菌株 GS115-MIT1 在含有甘油的培养基中均可以诱导 Aox1 的表达，野生菌株均不能诱导 Aox 的表达。

5、毕赤酵母 GFP 单拷贝表达菌株的筛选

在载体 pPIC3.5k (图 9) 的 *AOX1* 启动子下游的 *Sna*B I 酶切位点处插入 GFP 基因(全长 714 bp)，得到 GFP 表达载体 pP-GFP。

6、毕赤酵母 GFP 单拷贝表达菌株的筛选

分别将表达载体 pP-GFP 电转 GS115-MIT1 菌株，涂于不含组氨酸的 YND 平板，放在 30℃培养箱培养 48-72 小时。将平板上长出的单克隆挑至液体培养基中，30℃摇床培养后提基因组，用 real-time PCR 验证 GFP 拷贝数(方法参见 Xuan, Y. J., X. S. Zhou, W. W. Zhang, X. Zhang, Z. W. Song, and Y. X. Zhang. 2009. An upstream activation sequence controls the expression of *AOX1* gene in *Pichia pastoris*. FEMS Yeast Res. 9: 1271-1282)。把 real-time PCR 检验为单拷贝的毕赤酵母 GFP 表达菌株分别命名为 GS115-MIT1-GFP。

同理构建了表达菌株 Δ*mig1*Δ*mig2*-MIT1-GFP 和 Δ*hxs1*-MIT1-GFP。

表达菌株 Δ*mig1*Δ*mig2*-MIT1-GFP 构建方法具体如下：将表达载体 pP-GFP 和表达载体 pGMhph 共转 Δ*mig1*Δ*mig2* 菌株 (参见中国公开专利 CN101857845A)。

表达菌株 Δ*hxs1*-MIT1-GFP 构建方法具体如下：将表达载体 pP-GFP 和表达载体 pGMhph 共转 Δ*hxs1*-MIT1 菌株。

7、流式细胞仪检测 GFP 表达量

将菌株 GS115-MIT1-GFP 在 YND 液体培养基中过夜预培养，然后分别转移含有 0.5% (v/v) 甲醇，1%甘油，1%甘油+0.5% (v/v) 甲醇为碳源的 YNB 液体培养基中培养，取样后用流式细胞仪检测样品中 GFP 的几何平均荧光强度。

如图 5 所示，GS115-MIT1 可以在甘油存在的情况下诱导外源多肽 GFP 表达。

实施例 2、甘油或者葡萄糖可诱导 *AOX1* 启动子表达的毕赤酵母菌株

1. *HXS1* 基因敲除质粒的构建

用 *Bam*H I 和 *Sal* I 酶将 Zeocin 抗性基因 *Sh ble* 片段从质粒 pGAPZαA 上切下，以 pUC18 质粒(图 10)为载体，在 *Bam*H I 和 *Sal* I 处酶切连接插入了 Zeocin 抗性基因 *Sh ble* 片段，命名为 pUC18-*ble* 质粒。以 GS115 基因组为模板，首先使用引物 HS1-5F/HS1-5R 扩增 Pp*HXS1* 基因的 5'端外围启动子区域(起始密码子 ATG 上游)，扩增产物的大小为 728 bp。引物 HS1-3F/HS1-3R 用于扩增 Pp*HXS1* 基因的 3'端的区域(终止密码子下游)，扩增产物的大小为 1011 bp，分别切胶回收目的大小片段。

将回收的 Pp*HXS1* 基因的 5'端外围启动子 DNA 片段，用 *Eco*RI 和 *Bam*HI 双酶切后，回收片段。pUC18-*ble* 质粒、用 *Eco*RI 和 *Bam*HI 双酶切，回收片段。将回收的两片段连接过夜，转化大肠杆菌，PCR 验证阳性克隆，命名为 pUC18-(*HS1 5'-ble*)。

将上一步构建好质粒 *Sal*I 和 *Sph*I 双酶切，回收片段。Pp*HXS1* 基因的 3'端区域 DNA 片段也用 *Sal*I 和 *Sph*I 双酶切。两片段连接过夜，转化大肠杆菌，PCR 阳性菌株，阳性菌株命名为 pUC18-(*HS1 5'-ble-HS1 3'*)。

表 2、Pp*HXS1* 敲除使用的引物

引物名称	SEQ ID NO:	序列
HS1-5F	11	5'-CCGGAATTCTAGTCACGGATTGCTTC-3'
HS1-5R	12	5'-CGCGGATCCAGTCTGAAATAGGCTCAGACAT-3'
HS1-3F	13	5'-ACGCGTCGACATGCCTATTGAACAAGACTATT-3'
HS1-3R	14	5'-ACATGCATGCGTTCCCAATAGATTTCACAA-3'

2. Δ*hxs1* 基因缺失菌株的构建

将 pUC18-(*HS1 5'-ble-HS1 3'*)质粒用 *Eco*RI 和 *Sph*I 双酶切，胶回收大小为 3350 bp 的 Pp*HXS1* 敲除片段 *HS1 5'-ble-HS1 3'*。

将回收的 20 μl Pp*HXS1* 敲除片段 *HS1 5'-ble-HS1 3'*(浓度大于 50 ng/μl)与 80 μl 毕赤酵母 GS115 感受态在冰上混匀，按照实验步骤提供的方法进行电转化。将 50 μl 复苏的电转化菌液涂于添加 Zeocin 抗生素的 YP+Glycerol 固体培养基平板，将筛选平板倒置于 30℃培养箱培养 2～3 天，当有肉眼可见菌落长出时，将菌落挑至含 Zeocin 的 YP+Glycerol 液体培养基中培养 2～3 天。PCR 验证 Pp*HXS1* 敲除的阳性菌株，命名为 Δ*hxs1*。

3. Δ*mig1*Δ*mig2*-MIT1 和 Δ*hxs1*-MIT1 的构建

将质粒 pGMhph 电转毕赤酵母菌株 Δ*mig1*Δ*mig2* 和 Δ*hxs1*，涂于 4 块加潮霉素的 YPD 平板，放在 30℃培养箱培养 48-72 小时。将平板上长出的单克隆挑至 10 ml YPD+潮霉素液体培养基中，30℃摇床培养后，抽提基因组用 PCR 验证。把 PCR 检验及测序验证后正确的毕赤酵母菌株分别命名为 Δ*mig1*Δ*mig2*-MIT1 和 Δ*hxs1*-MIT1。

4. Aox 显色反应

将 Δ*mig1*Δ*mig2*-MIT1 和 Δ*hxs1*-MIT1 分别在 1% 甘油，1% 甘油+0.5% (v/v) 甲醇，1% 葡萄糖，1% 葡萄糖+0.5% (v/v) 甲醇和 0.5% (v/v) 甲醇为碳源的 YNB 液体培养基中培养，生长到对数生长期后取样 1ml，在离心后的菌体中加入 Aox1 酶活显色液显色。

结果如图 6，可以看到在含有甘油或者葡萄糖的培养基中，只有菌株 Δ*mig1*Δ*mig2*-MIT1 和 Δ*hxs1*-MIT1 中有酶活。

5. Aox 酶活的测定

将各菌株分别在 1% 甘油，1%甘油+0.5% (v/v) 甲醇，1% 葡萄糖，1% 葡萄糖+0.5% (v/v) 甲醇和 0.5% (v/v) 甲醇为碳源的 YNB 液体培养基中培养，生长到对数生长期后取样提取总蛋白，经 Bradford 法蛋白定量后进行 Aox1 酶活的测定。

由图 7 可知，菌株 Δ*mig1*Δ*mig2*-MIT1 和 Δ*hxs1*-MIT1 在含有甘油或者葡萄糖的培养基中均可以诱导 Aox1 的表达，野生菌株均不能诱导 Aox 的表达。

6. 流式细胞仪检测 Δ*mig1*Δ*mig2*-MIT1 和 Δ*hxs1*-MIT1 的 GFP 表达量

将 Δ*mig1*Δ*mig2*-MIT1-GFP 和 Δ*hxs1*-MIT1-GFP 在液体培养基中过夜预培养，然后分别转移到含有 0.5% (v/v) 甲醇，1% 甘油，1% 甘油+0.5% (v/v) 甲醇，1%

葡萄糖和 1% 葡萄糖+0.5% (v/v) 甲醇为碳源的 YNB 液体培养基中，取样后用流式细胞仪检测样品中 GFP 的几何平均荧光强度。

如图 8 所示，Δ*mig1*Δ*mig2*-MIT1 和 Δ*hxs1*-MIT1 可以在甘油或者葡萄糖存在的情况下诱导外源多肽 GFP 表达。

实施例 5、甘油或者葡萄糖可诱导 *AOX1* 启动子表达的毕赤酵母菌株 Δ*mig1*Δ*mig2*Δ*nrg1*-MIT1

1. *NRG1* 基因敲除质粒的构建

以 GS115 基因组为模板，使用引物 5'NRG1-F/5'NRG1-R 扩增 *PpNIG1* 基因的 5'端外围区域得到大小为 320 bp 片段，将扩增产物命名为 5'*NRG1*，胶回收 5'*NRG1* 片段。以上所得到的 5'*NRG1* 片段用 *Sac* I 和 *Sma* I 双酶切，与同样 *Sac* I 和 *Sma* I 双酶切的 pUC18 质粒连接，构成 pUC18(*Sac*I-5'NRG1-*Sma*I)。载体 pUC18(*Sac*I-5'NRG1-*Sma*I)转化大肠杆菌感受态，菌落 PCR 筛选阳性克隆。

然后，使用引物 HYG-F 和 HYG-R，以质粒 pRDM054 为模板，扩增潮霉素 B 抗性基因 *HPH*，大小为 1648 bp。以上所得 *HPH* 片段用 *Sma* I 和 *Xba*I 双酶切，与同样 *Sma* I 和 *Xba*I 双酶切的载体 pUC18(*Sac*I-5'NRG1-*Sma*I)相连，构成载体 pUC18(*Sac*I-5'NRG1-*Sma*I-HPH-*Xba*I)。pUC18(*Sac*I-5'NRG1-*Sma*I- HPH-*Xba*I)转化入大肠杆菌，菌落 PCR 筛选阳性克隆。

用引物 5'NRG1-F/NRG1-A1 以质粒 pUC18(*Sac*I-5'NRG1-*Sma*I-HPH-*Xba*I)为模板扩增得到片段 Overlap-A。用引物 NRG1-B2/3'NRG1-R，以 GS115 基因组为模板，扩增得到片段 Overlap-B。然后通过 Overlap PCR 的方法链接片段 A、B，得到 5'NRG1-HPH-3'NRG1 片段。将该片段连接到 pMD19-T 载体上，得到敲除质粒 pMD19-T(*Sac*I-5'NRG1-*Sma*I-HPH-3'NRG1-*Sph*I)。

2. Δ*mig1*Δ*mig2*Δ*nrg1* 菌株的构建

将得到的 *Sac*I-5'NRG1-*Sma*I-HPH-3'NRG1-*Sph*I 片段与 Δ*mig1*Δ*mig2* 双缺失菌株感受态共转，电转后迅速加入 700 ul 预冷的 1 mol/L 山梨醇，然后转移至 EP 管中，并加入 700 ul YPD 液体培养基，30℃和 200 r/min 摇床复苏培养 1～2 h。最后将菌液涂于添加 Zeocin、G418 和 Hygromycin 抗生素的 YPD 固体平板。30℃培养箱倒置培养 2～3 天。将阳性转化子提基因组 PCR 验证，得到的阳性菌株命名为 Δ*mig1*Δ*mig2*Δ*nrg1*。

3. 三缺失菌株中 MIT1 过表达质粒的构建

以 pPIC3.5k 为载体在 *Sac* I/*Bam*H I 两个酶切位点插入 GAP 启动子得到 pPG 质粒。再于 BamH I/Not I 两个酶切位点间插入 PpMPP1 基因序列，得到的重组质粒称为 pPGPP1 质粒。

4. Δ*mig1*Δ*mig2*Δ*nrg1*-MIT1 的构建

将质粒 pPGPP1 电转毕赤酵母菌株 Δ*mig1*Δ*mig2*Δ*nrg1*，涂于 4 块不加 his 的 YND 平板，放在 30℃培养箱培养 48-72 小时。将平板上长出的单克隆挑至 10 ml YND 液体培养基中，30℃摇床培养后，抽提基因组用 PCR 验证。把 PCR 检验及测序验证后正确的毕赤酵母菌株分别命名为 Δ*mig1*Δ*mig2*Δ*nrg1*-MIT1。

5. 毕赤酵母 GFP 单拷贝表达菌株的筛选

分别将表达载体 pP-GFP 电转 GS11+-MIT1 菌株，涂于不含组氨酸的 YND 平板，放在 30℃培养箱培养 48-72 小时。将平板上长出的单克隆挑至 MGY 液体培养基中，在荧光显微镜下观察是否有绿色荧光，有荧光的转化子 30℃摇床培养后提基因组，用 real-time PCR 验证 GFP 拷贝数(方法参见 Xuan, Y. J., X. S. Zhou, W. W. Zhang, X. Zhang, Z. W. Song, and Y. X. Zhang. 2009. An upstream activation sequence controls the expression of *AOX1* gene in *Pichia pastoris*. FEMS Yeast Res. 9: 1271–1282)。把 real-time PCR 检验为单拷贝的毕赤酵母 GFP 表达菌株命名为 Δ*mig1*Δ*mig2*Δ*nrg1*-MIT1-GFP。

6. Aox 酶活的测定

将 Δ*mig1*Δ*mig2*Δ*nrg1*-MIT1 菌株分别在 1% 甘油，1%甘油+0.5% (v/v) 甲醇，1% 葡萄糖，1% 葡萄糖+0.5% (v/v) 甲醇和 0.5% (v/v) 甲醇为碳源的 YNB 液体培养基中培养，生长到对数生长期后取样提取总蛋白，经 Bradford 法蛋白定量后进行 Aox1 酶活的测定。

由图 11 可知，菌株 Δ*mig1*Δ*mig2*Δ*nrg1*-MIT1 在含有甘油或者葡萄糖的培养基中均可以诱导 Aox1 的表达，野生菌株均不能诱导 Aox 的表达。

7. 流式细胞仪检测 Δ*mig1*Δ*mig2*Δ*nrg1*-MIT1 的 GFP 表达量

将 Δ*mig1*Δ*mig2*Δ*nrg1*-MIT1-GFP 在液体培养基中过夜预培养，然后分别转移到含有 0.5% (v/v) 甲醇，1% 甘油，1% 甘油+0.5% (v/v) 甲醇，1% 葡萄糖和 1% 葡萄糖+0.5% (v/v) 甲醇为碳源的 YNB 液体培养基中，取样后用流式细胞仪检测样品中 GFP 的几何平均荧光强度。

如图 12 所示，Δ*mig1*Δ*mig2*Δ*nrg1*-MIT1 可以在甘油或者葡萄糖存在的情况下诱导外源多肽 GFP 表达。

表 3、*PpNIG1* 敲除使用的引物

引物名称	SEQ ID NO:	序列
5'NRG1-F	15	5'- CGAGCTCCTGTGCCTATTACCCCCCTT -3'
5'NRG1-R	16	5'- TCCCCCGGGAACAGATAACCAAAACGGACG -3'
3'NRG1-F	17	5- GCTCTAGAGTATTTATTTACGGATTGGA -3'
3'NRG1-R	18	5'- ACATGCATGCCACCACTTTTTGAATCTCGG -3'
HYG-F	19	5'- TCCCCCGGGAGCTTGCCTTGTCCCCGCCG -3'
HYG-R	20	5'- GCTCTAGATCGACACTGGATGGCGGCGT -3'
NRG1-A1	21	5'-TCCAATCCGTAAATAAATACTCGACACTGGATGGCGGCGT-3'
NRG1-B2	22	5'-ACGCCGCCATCCAGTGTCGAGTATTTATTTACGGATTGGA-3'

在本发明提及的所有文献都在本申请中引用作为参考，就如同每一篇文献被单独引用作为参考那样。此外应理解，在阅读了本发明的上述讲授内容之后，本领域技术人员可以对本发明作各种改动或修改，这些等价形式同样落于本申请所附权利要求书所限定的范围。

权 利 要 求

1. 一种消除甲醇诱导型启动子单一甲醇碳源依赖性而驱动外源多肽编码基因表达的方法，包括：

(1) 提供甲醇营养型酵母，所述甲醇营养型酵母含有：

表达盒 1，其表达外源的 Mit1 多肽；以及

表达盒 2，包括甲醇诱导型启动子及与之操作性连接的外源多肽编码基因；

(2) 在无甲醇条件或非单一甲醇碳源条件下培养(1)的甲醇营养型酵母。

2. 如权利要求 1 所述的方法，其特征在于，所述的甲醇诱导型启动子包括：*AOX1* 启动子、*DHAS* 启动子、*DAS* 启动子、*FDH* 启动子、*FMDH* 启动子、*MOX* 启动子、*AOX2* 启动子、*ZZA1*、*PEX5*-、*PEX8*-、*PEX14*-启动子、*PMP20* 启动子、*PMP47* 启动子、*AOD1* 启动子、*AOD2* 启动子。

3. 如权利要求 1 所述的方法，其特征在于，所述的甲醇营养型酵母包括：毕赤酵母(*Pichia*)，汉逊酵母(*Hansenula*)，假丝酵母(*Candida*)，球拟酵母(*Torulopsis*)；较佳地，所述的毕赤酵母包括：GS115，*MIG1* 基因和 *MIG2* 基因不表达的毕赤酵母，*NRG1* 基因、*MIG1* 基因和 *MIG2* 基因不表达的毕赤酵母，或 *HXS1* 基因不表达的毕赤酵母菌株。

4. 如权利要求 1 所述的方法，其特征在于，所述的 Mit1 多肽选自下组：

(a) SEQ ID NO: 2 所示氨基酸序列的多肽；或

(b) 将 SEQ ID NO: 2 所示氨基酸序列经过一个或多个氨基酸残基的取代、缺失或添加而形成的，且具有利用非甲醇碳源诱导甲醇诱导型启动子表达外源多肽功能的由(a)衍生的多肽；

(c) 与(a)限定的序列在具有 70%以上的序列相同性的，且具有利用非甲醇碳源诱导甲醇诱导型启动子表达外源多肽功能的由(a)衍生的多肽。

5. 如权利要求 1 所述的方法，其特征在于，所述的表达盒 1 中，包括启动子以及与之操作性连接的 Mit1 多肽的编码基因；较佳地，该启动子包括：组成型启动子，诱导型启动子，组织或器官特异性启动子，时空特异性表达启动子。

6. 如权利要求 1 所述的方法，其特征在于，步骤(2)中，采用存在甘油和/或葡萄糖的酵母培养基。

7. Mit1 多肽或其编码基因的用途，用于消除甲醇诱导型启动子单一甲醇碳源依赖性而驱动外源多肽编码基因表达。

8. 一种重组的甲醇营养型酵母，所述甲醇营养型酵母含有：

表达盒 1，其能够表达外源的 Mit1 多肽；以及

表达盒 2，包括甲醇诱导型启动子及与之操作性连接的外源多肽编码基因。

9. 如权利要求 8 所述的重组的甲醇营养型酵母，其特征在于，所述的甲醇营养型酵母包括：毕赤酵母(*Pichia*)，汉逊酵母(*Hansenula*)，假丝酵母(*Candida*)，球拟酵母(*Torulopsis*)；较佳地，所述的毕赤酵母(*Pichia*)包括：GS115，*MIG1* 基因和 *MIG2* 基因不表达的毕赤酵母，*NRG1* 基因、*MIG1* 基因和 *MIG2* 基因不表达的毕赤酵母，或 *HXS1* 基因不表达的毕赤酵母菌株。

10. 如权利要求 9 所述的方法，其特征在于，所述的 *MIG1* 基因的核苷酸序列如 SEQ ID NO: 9 所示；

所述的 *MIG2* 基因的核苷酸序列如 SEQ ID NO: 10 所示；或

所述的 *HXS1* 基因的核苷酸序列如 SEQ ID NO: 3 所示。

所述的 *NRG1* 基因的核苷酸序列如 SEQ ID NO:8 所示。

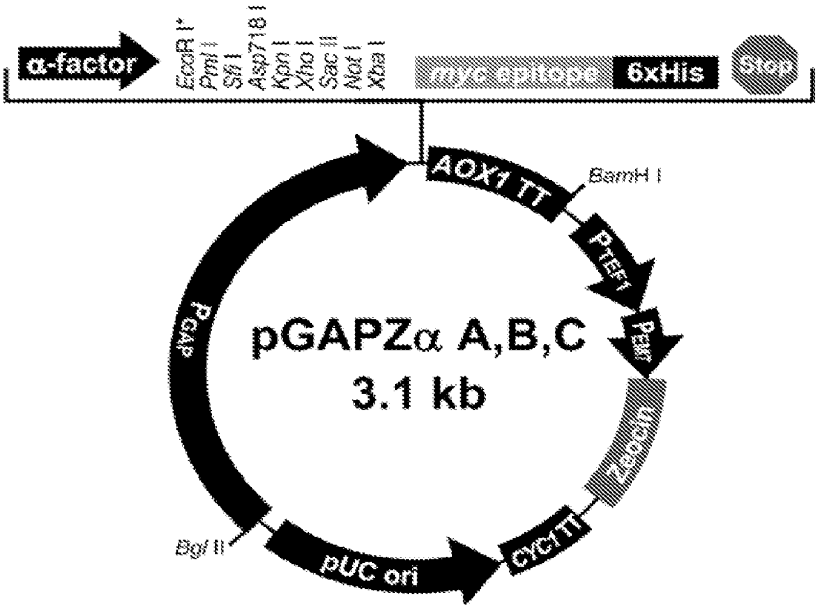

α-factor
EcoR I*
Pml I
Sfi I
Asp718 I
Kpn I
Xho I
Sac II
Not I
Xba I
myc epitope
6xHis
Stop
AOX1 TT
BamH I
PTEF1
Zeocin
CYC1 TT
pUC ori
Bgl II
PGAP
pGAPZα A,B,C
3.1 kb

图 1

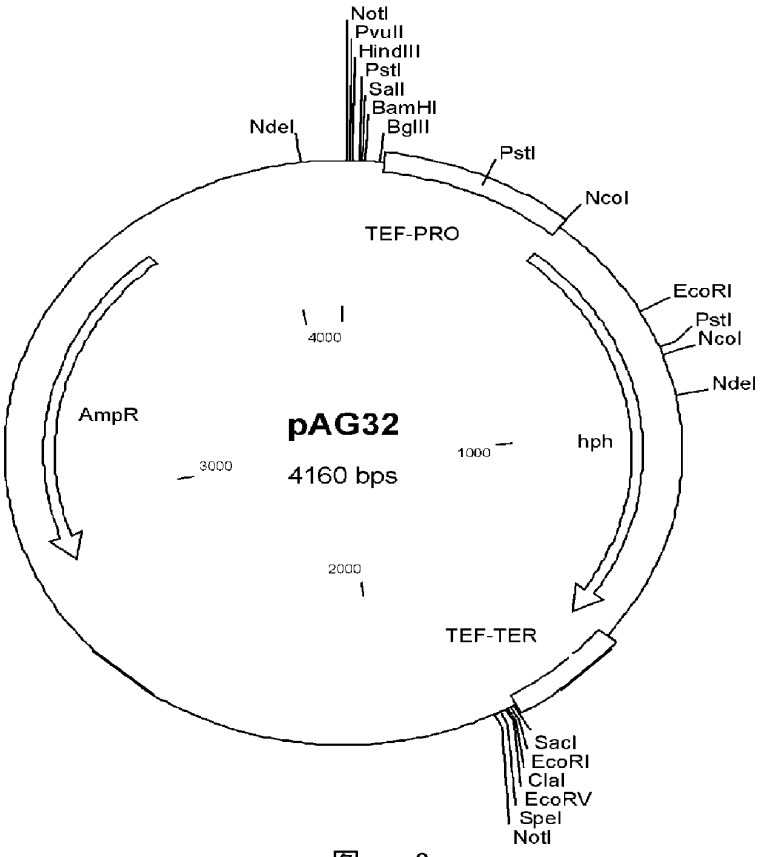

NotI
PvuII
HindIII
PstI
SalI
BamHI
BglII
NdeI
PstI
NcoI
TEF-PRO
EcoRI
PstI
NcoI
NdeI
4000
AmpR
pAG32
4160 bps
1000
hph
3000
2000
TEF-TER
SacI
EcoRI
ClaI
EcoRV
SpeI
NotI

图 2

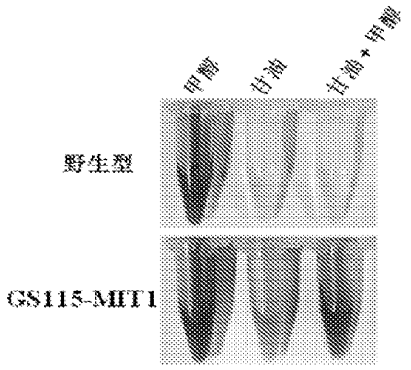

甲醇
甘油
甘油+甲醇
野生型
GS115-MIT1

图 3

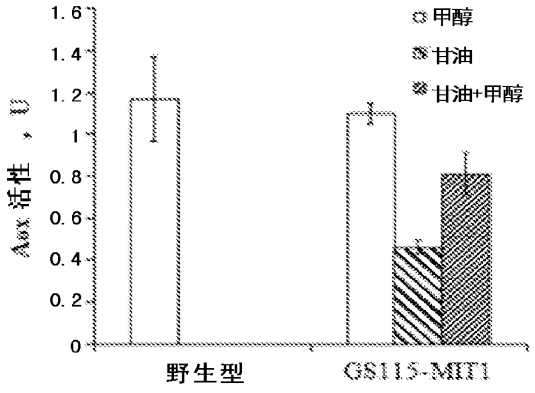

甲醇
甘油
甘油+甲醇
Aox 活性，U
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
野生型
GS115-MIT1

图 4

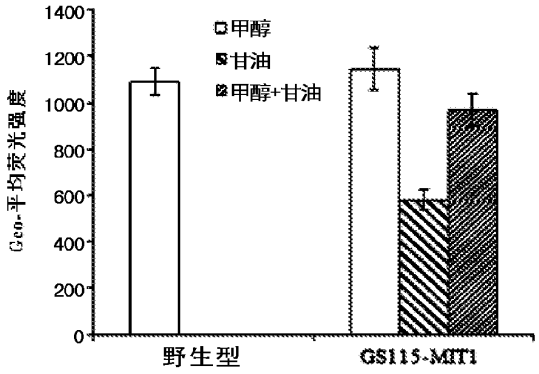

甲醇
甘油
甲醇+甘油
Geo-平均荧光强度
1400
1200
1000
800
600
400
200
0
野生型
GS115-MIT1

图 5

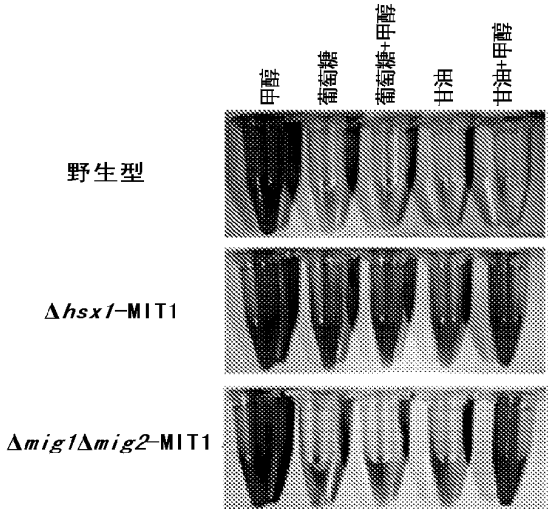

甲醇
葡萄糖
葡萄糖+甲醇
甘油
甘油+甲醇
野生型
Δhsx1-MIT1
Δmig1Δmig2-MIT1

图 6

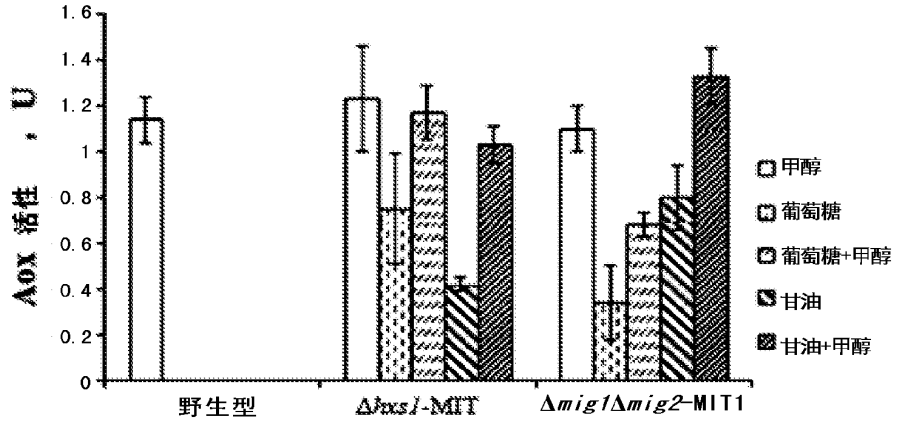

Aox 活性，U
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
甲醇
葡萄糖
葡萄糖+甲醇
甘油
甘油+甲醇
野生型
Δhxs1-MIT
Δmig1Δmig2-MIT1

图 7

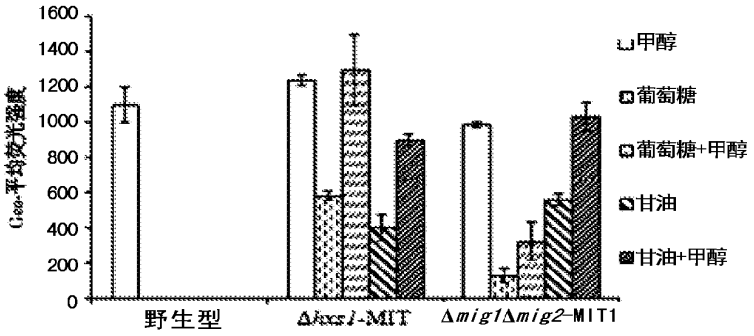


图 8

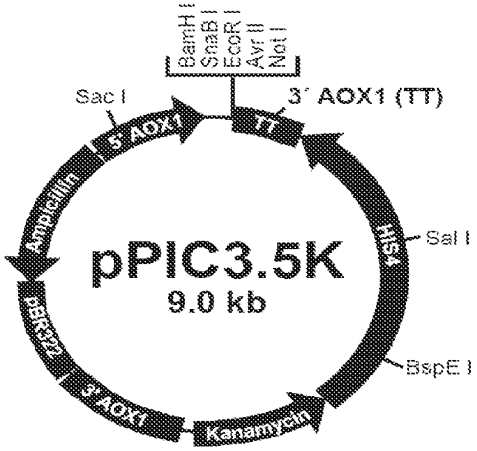


图 9

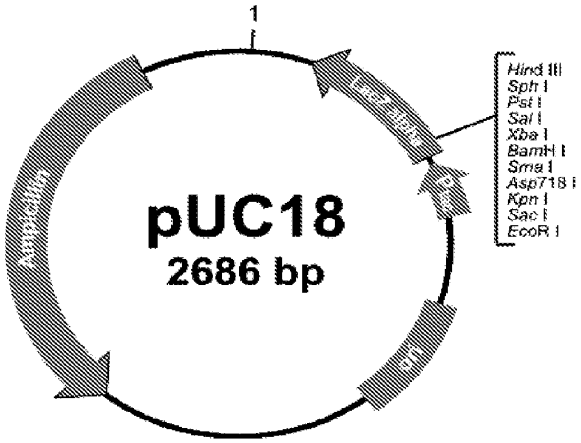


图 10

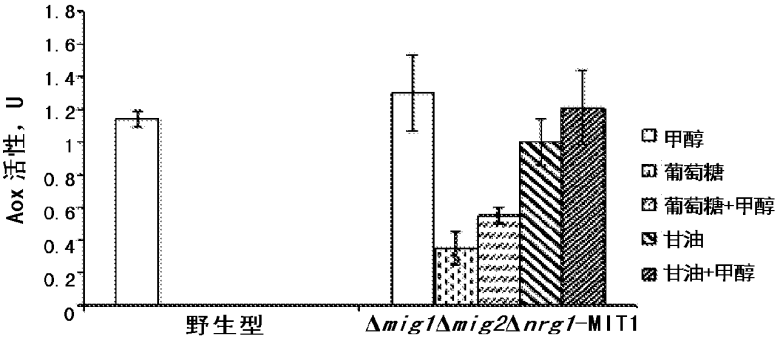


图 11

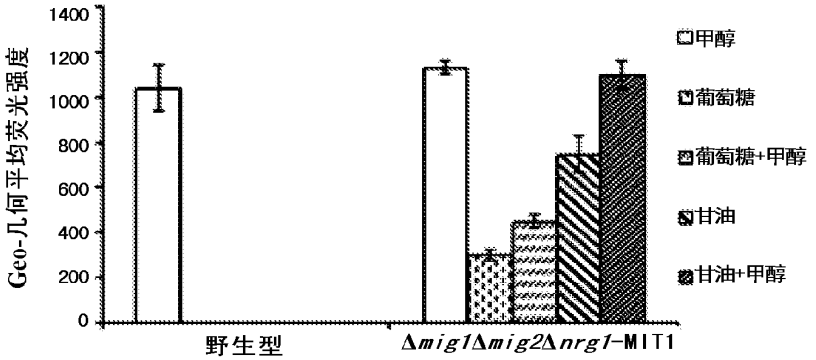


图 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2012/084642

A. CLASSIFICATION OF SUBJECT MATTER

See the extra sheet

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: C12N, C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Data bases: CNABS, CPRSABS, DWPI, CPEA, SIPOABS, CNTXT, EPTXT, USTXT, WOTXT, CJFD, CNKI, ISI WEB OF KNOWLEDGE, GenBANK+EMBL+DDBJ

Search terms: transcription activating related factor, containment, carbon source, mitl, methanol, yeast, Pichia, methanol induced transcription factor, induc+, promoter, glycerol, methanol inducible promoter, eliminate+, replac+, substitute+, remov+, sequence search on sequences 1 and 2

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	CN 102757976 A (EAST CHINA UNIVERSITY OF SCIENCE AND TECHNOLOGY), 31 October 2012 (31.10.2012), see claims 1-10	1-10
A	CN 101589146 A (NOVOZYMES A/S), 25 November 2009 (25.11.2009), see claims 1-19, and description, pages 9 and 18	1-10
A	CN 101857845 A (EAST CHINA UNIVERSITY OF SCIENCE AND TECHNOLOGY), 13 October 2010 (13.10.2010), see the whole document	1-10

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
07 April 2013 (07.04.2013)	**18 April 2013 (18.04.2013)**
Name and mailing address of the ISA/CN: State Intellectual Property Office of the P. R. China No. 6, Xitucheng Road, Jimenqiao Haidian District, Beijing 100088, China Facsimile No.: (86-10) 62019451	Authorized officer **WANG, Huimei** Telephone No.: (86-10) **62411090**

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2012/084642

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☒ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2012/084642

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LIN-CEREGHINO, G.P. et al., "Mxrlp, a key regulator of the methanol Utilization pathway and peroxisomal genes in pichia pastoris", MOLECULAR AND CELLULAR BIOLOGY, vol. 26, no. 3, pages 883-897, February 2006, see the whole document	1-10
A	DE, S.K., "Pichia pastoris GS115 hypothetical protein (PAS_chr3_0836) mRNA, complete cds", GenBank accession number: XM_002493021, 22 July 2009 (22.07.2009), see the whole document	4
A	CAI, Mengnan, "Glycerol-limitation-induced production of recombinant proteins in Hansenula polymorpha", MASTER'S DEGREE THESIS OF INSTITUTE OF MICROBIOLOGY AND BIOCHEMISTRY, NATIONAL TAIWAN UNIVERSITY, 11 May 2010 (11.05.2010), see the whole document	1-10
A	YING, Guoxin; "Screening of the Differentially Expressed Genes and Cystatin C Expression in the Deafferented Hippocampus", CHINA DOCTORAL DISSERTATIONS FULL-TEXT DATABASE (BASIC SCIENCES), ISSN 1671-6779, 2003, no. 2, 20 July 2003 (20.07.2003), see page 32	1-10

Form PCT/ISA/210 (continuation of second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2012/084642

Patent Documents referred in the Report	Publication Date	Patent Family	Publication Date
CN 102757976 A	31.10.2012	None	
CN 101589146 A	25.11.2009	US 2012142053 A1	07.06.2012
		US 2009311749 A1	17.12.2009
		WO 2008090211 A1	31.07.2008
		JP 2010516268 A	20.05.2010
		US 8143023 B2	27.03.2012
		US 8236528 B2	07.08.2012
		IN 200904439 P4	02.10.2009
		EP 2106447 A1	07.10.2009
CN 101857845 A	13.10.2010	None	

Form PCT/ISA/210 (patent family annex) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2012/084642

A. CLASSIFICATION OF SUBJECT MATTER:

IPC:

C12N 15/81 (2006.01) i

C12N 1/19 (2006.01) i

C12R 1/84 (2006.01) n

C12R 1/78 (2006.01) n

C12R 1/72 (2006.01) n

C12R 1/88 (2006.01) n

国际检索报告

国际申请号
PCT/CN2012/084642

A. 主题的分类

参见附加页

按照国际专利分类(IPC)或者同时按照国家分类和 IPC 两种分类

B. 检索领域

检索的最低限度文献(标明分类系统和分类号)

IPC: C12N, C12R

包含在检索领域中的除最低限度文献以外的检索文献

在国际检索时查阅的电子数据库(数据库的名称，和使用的检索词（如使用）)

数据库：CNABS, CPRSABS, DWPI, CPEA, SIPOABS, CNTXT, EPTXT, USTXT, WOTXT, CJFD, CNKI, ISI WEB OF KNOWLEDGE，GenBANK +EMBL+DDBJ

检索词：甲醇，酵母，毕赤酵母，转录激活相关因子，甲醇诱导的转录因子，甘油，启动子，诱导，消除，遏制，取代，代替，碳源，甲醇诱导型启动子，mit1, methanol, yeast, Pichia, methanol induced transcription factor, induc+, promoter, glycerol, methanol inducible promoter, eliminate+, replac+,substitute+, remov+，关于序列 1 和 2 的序列检索

C. 相关文件

类　型*	引用文件，必要时，指明相关段落	相关的权利要求
PX	CN102757976A （华东理工大学），31.10 月 2012（31.10.2012），参见权利要求 1-10	1-10
A	CN101589146A（诺维信公司），25.11 月 2009（25.11.2009），参见权利要求 1-19，说明书第 9 和 18 页	1-10
A	CN101857845A（华东理工大学），13.10 月 2010（13.10.2010），参见全文	1-10

☒ 其余文件在 C 栏的续页中列出。　　☒ 见同族专利附件。

* 引用文件的具体类型：
"A" 认为不特别相关的表示了现有技术一般状态的文件
"E" 在国际申请日的当天或之后公布的在先申请或专利
"L" 可能对优先权要求构成怀疑的文件，或为确定另一篇引用文件的公布日而引用的或者因其他特殊理由而引用的文件（如具体说明的）
"O" 涉及口头公开、使用、展览或其他方式公开的文件
"P" 公布日先于国际申请日但迟于所要求的优先权日的文件
"T" 在申请日或优先权日之后公布，与申请不相抵触，但为了理解发明之理论或原理的在后文件
"X" 特别相关的文件，单独考虑该文件，认定要求保护的发明不是新颖的或不具有创造性
"Y" 特别相关的文件，当该文件与另一篇或者多篇该类文件结合并且这种结合对于本领域技术人员为显而易见时，要求保护的发明不具有创造性
"&" 同族专利的文件

国际检索实际完成的日期	国际检索报告邮寄日期
07.4 月 2013（07.04.2013）	**18.4 月 2013 (18.04.2013)**
ISA/CN 的名称和邮寄地址： 中华人民共和国国家知识产权局 中国北京市海淀区蓟门桥西土城路 6 号 100088 传真号：　(86-10)62019451	受权官员 王慧梅 电话号码：(86-10) **62411090**

PCT/ISA/210 表(第 2 页) (2009 年 7 月)

国际检索报告

国际申请号
PCT/CN2012/084642

第I栏　核苷酸和/或氨基酸序列(续第 1 页第 1.c 项)

1、关于国际申请中所公开的任何对要求保护的发明必要的**核苷酸和/或氨基酸序列**, 国际检索是在下列基础上进行的：

a. 序列表的提交或提供

☐ 纸件形式

☒ 电子形式

b. 提交或提供时间

☒ 包含在申请提交时的国际申请中

☐ 以电子形式与国际申请一起提交

☐ 为检索之用随后提交本单位

2、☐ 另外，在提交/提供了多个版本或副本的序列表的情况下，提供了关于随后提交的或附加的副本中的信息与申请时提交的申请中的信息相同或未超出申请时提交的申请中的信息范围（如适用）的所需声明。

3. 补充意见

国际检索报告

国际申请号
PCT/CN2012/084642

C(续). 相关文件

类　型	引用文件，必要时，指明相关段落	相关的权利要求
A	Geoffrey Paul Lin-Cereghino 等，“Mxr1p, a key regulator of the methanol Utilization pathway and peroxisomal genes in pichia pastoris”，Molecular AND CELLULAR BIOLOGY，第 26 卷，第 3 期，第 883-897 页，2006 年 2 月，参见全文	1-10
A	De Schutter,K.，“Pichia pastoris GS115 hypothetical protein (PAS_chr3_0836) mRNA, complete cds”，GenBank 登录号：XM_002493021，22.7 月 2009（22.07.2009），参见全文	4
A	蔡孟男，“利用甘油受限诱导汉逊氏酵母菌表现重组蛋白质”，台湾大学微生物与生化学研究所硕士论文，11.5 月 2010（11.05.2010），参见全文	1-10
A	应国新，“去传入海马的差异表达基因的筛选和 Cystatin C 表达”，中国优秀博士学位论文全文数据库（基础科学辑），ISSN 1671-6779，2003 年第 2 期，20.7 月 2003（20.07.2003），参见第 32 页	1-10

国际检索报告
关于同族专利的信息

国际申请号
PCT/CN2012/084642

检索报告中引用的专利文件	公布日期	同族专利	公布日期
CN102757976A	31.10.2012	无	
CN101589146A	25.11.2009	US2012142053 A1	07.06.2012
		US2009311749 A1	17.12.2009
		WO2008090211 A1	31.07.2008
		JP2010516268 A	20.05.2010
		US8143023 B2	27.03.2012
		US8236528 B2	07.08.2012
		IN200904439P4	02.10.2009
		EP2106447 A1	07.10.2009
CN101857845A	13.10.2010	无	

PCT/ISA/210 表(同族专利附件) (2009 年 7 月)

国际检索报告

国际申请号
PCT/CN2012/084642

A．主题的分类

IPC:

C12N 15/81(2006.01)i

C12N 1/19(2006.01)i

C12R 1/84(2006.01)n

C12R 1/78(2006.01)n

C12R 1/72(2006.01)n

C12R 1/88(2006.01)n

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter I of the Patent Cooperation Treaty)

(PCT Rule 44*bis*)

Applicant's or agent's file reference 38767004SWO1	**FOR FURTHER ACTION**	See item 4 below
International application No. PCT/US2016/031797	International filing date *(day/month/year)* 11 May 2016 (11.05.2016)	Priority date *(day/month/year)* 11 May 2015 (11.05.2015)
International Patent Classification (8th edition unless older edition indicated) See relevant information in Form PCT/ISA/237		
Applicant IMPOSSIBLE FOODS INC.		

1. This international preliminary report on patentability (Chapter I) is issued by the International Bureau on behalf of the International Searching Authority under Rule 44 *bis*.1(a).

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   In the attached sheets, any reference to the written opinion of the International Searching Authority should be read as a reference to the international preliminary report on patentability (Chapter I) instead.

3. This report contains indications relating to the following items:

☒	Box No. I	Basis of the report
☐	Box No. II	Priority
☒	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☐	Box No. VI	Certain documents cited
☐	Box No. VII	Certain defects in the international application
☐	Box No. VIII	Certain observations on the international application

4. The International Bureau will communicate this report to designated Offices in accordance with Rules 44*bis*.3(c) and 93*bis*.1 but not, except where the applicant makes an express request under Article 23(2), before the expiration of 30 months from the priority date (Rule 44*bis* .2).

	Date of issuance of this report 14 November 2017 (14.11.2017)
The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. +41 22 338 82 70	Authorized officer Kihwan Moon e-mail: pct.team1@wipo.int

Form PCT/IB/373 (January 2004)

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To: M. Angela Parsons
Fish & Richardson P.C.
P.O. Box 1022
Minneapolis, Minnesota 55440-1022
United States of America

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing *(day/month/year)*	20 SEP 2016
Applicant's or agent's file reference 387670048WO1	FOR FURTHER ACTION See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US16/31797	11 May 2016 (11.05.2016)	11 May 2015 (11.05.2015)

International Patent Classification (IPC) or both national classification and IPC
IPC(8) - C07K 14/39; C12N 15/00, 15/81; C12R 1/84 (2016.01)
CPC - C07K 14/39; C12N 15/00, 15/81; C12R 1/84

Applicant IMPOSSIBLE FOODS INC.

1. This opinion contains indications relating to the following items:

- [X] Box No. I Basis of the opinion
- [ ] Box No. II Priority
- [X] Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- [ ] Box No. IV Lack of unity of invention
- [X] Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- [ ] Box No. VI Certain documents cited
- [ ] Box No. VII Certain defects in the international application
- [ ] Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/	Date of completion of this opinion	Authorized officer
Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	29 August 2016 (29.08.2016)	Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/237 (cover sheet) (January 2015)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US16/31797

Box No. I Basis of this opinion

1. With regard to the language, this opinion has been established on the basis of:

☒ the international application in the language in which it was filed.

☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. ☐ This opinion has been established taking into account the rectification of an obvious mistake authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a)).

3. ☒ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this opinion has been established on the basis of a sequence listing:

a. ☐ forming part of the international application as filed:

☐ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

4. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US16/31797

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 5-29, 35-38

because:

☐ the said international application, or the said claims Nos. _____ relate to the following subject matter which does not require an international search *(specify)*:

☒ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. 5-29, 35-38 are so unclear that no meaningful opinion could be formed *(specify)*:

Claims Nos.: 5-29, 35-38 are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

☐ the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed *(specify)*:

☒ no international search report has been established for said claims Nos. 5-29, 35-38

☐ a meaningful opinion could not be formed without the sequence listing; the applicant did not, within the prescribed time limit:

☐ furnish a sequence listing in the form of an Annex C/ST.25 text file, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.

☐ furnish a sequence listing on paper or in the form of an image file complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.

☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13*ter*.1(a) or (b).

☐ See Supplemental Box for further details.

Form PCT/ISA/237 (Box No. III) (January 2015)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US16/31797

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	30-32, 33/30-32, 34/30-32	YES
	Claims	1-2, 3/1-2, 4/1-2	NO
Inventive step (IS)	Claims	NONE	YES
	Claims	1-2, 3/1-2, 4/1-2, 30-32, 33/30-32, 34/30-32	NO
Industrial applicability (IA)	Claims	1-2, 3/1-2, 4/1-2, 30-32, 33/30-32, 34/30-32	YES
	Claims	NONE	NO

2. Citations and explanations:

Claims 1-2, 3/1-2, and 4/1-2 lack novelty under PCT Article 33(2) as being anticipated by WO 2014/008729 A1 (EAST CHINA UNIVERSITY OF SCIENCE AND TECHNOLOGY) (hereinafter 'China').

As per claim 1, China discloses a methylotrophic yeast cell (abstract) comprising a recombinant nucleic acid molecule (page 6, paragraph 6), wherein the recombinant nucleic acid molecule comprises an exogenous nucleic acid encoding a transcriptional activator (an exogenous gene encoding a methanol inducible transcription factor 1 (Mit1) polypeptide (a transcriptional activator); page 4, paragraph 6; page 7, paragraph 13; claim 1) operably linked to at least one methanol-inducible promoter element (operatively connected (linked) to a methanol inducible promoter; claim 1).

As per claim 2, China discloses the yeast cell of claim 1, and China further discloses wherein the methylotrophic yeast cell is of a yeast selected from the group consisting of Candida (page 2, paragraph 15), Hansenula (page 2, paragraph 15), Pichia (page 2, paragraph 15) and Torulopsis (page 2, paragraph 15).

As per claims 3/1-2, China discloses the yeast cell of claims 1-2, and China further discloses wherein the methylotrophic yeast cell is of a Pichia yeast (page 2, paragraph 15).

As per claims 4/1-2, China discloses the yeast cell of claims 1-2, and China further discloses wherein the cell is a Pichia cell (abstract; page 8, paragraphs 11-12).

Claims 30-32, 33/30-32, and 34/30-32 lack an inventive step under PCT Article 33(3) as being obvious over the publication entitled, 'Mxr1p, A Key Regulator Of The Methanol Utilization Pathway And Peroxisomal Genes in Pichia Pastoris', by Lin-Cereghino, GP et al. (hereinafter 'Lin-Cereghino') in further view of the publication entitled, 'GRAS Notification for Soybean Leghemoglobin Protein Derived from Pichia pastoris' by Impossible Foods Inc. (hereinafter 'Impossible'), and EP 2058396 A1 (Nipro Corporation) (hereinafter 'Nipro').

As per claim 30, Lin-Cereghino discloses a methylotrophic Pichia yeast cell (Pichia pastoris that grows on methanol (a methylotrophic Pichia yeast cell); abstract) comprising a recombinant nucleic acid molecule (abstract; page 886, column 2, paragraph 2), wherein the recombinant nucleic acid molecule comprises: a nucleic acid encoding a Mxr1 transcriptional activator sequence from P pastoris (a nucleic acid encoding MXR1; abstract; page 886, column 2, paragraph 2). Lin-Cereghino does not disclose a nucleic acid encoding a member of the globin family (PF00042); and a nucleic acid encoding at least one polypeptide involved in heme biosynthesis. Impossible discloses a nucleic acid encoding a member of the globin family (PF00042) (a leghemoglobin protein derived from Pichia pastoris (a member of the globin family (PF00042)); page 1, paragraph 1). It would have been obvious to a person of ordinary skill in the art, at the time the invention was made, to have modified the methylotrophic Pichia yeast cell, as previously disclosed by Lin-Cereghino, to include a nucleic acid encoding a member of the globin family (PF00042), as previously disclosed by Impossible, as the previous disclosure by Lin-Cereghino includes a method to improve growth of Pichia yeast cell on a growth medium to enhance the production of recombinant proteins (Lin-Cereghino; abstract; page 883, column 2, paragraph 2). Impossible discloses a soybean protein produced in Pichia yeast cells (Impossible, page 1, paragraph 1), and a combination of these references would have resulted in a superior methylotrophic Pichia yeast cell for producing a member of the globin family. Nipro discloses a nucleic acid encoding at least one polypeptide involved in heme biosynthesis (a DNA encoding a human hemoglobin α-chain (Hba), (a nucleic acid encoding at least one polypeptide involved in heme biosynthesis) which is under regulation of a promoter functional in a yeast of the genus Pichia; abstract). It would have been obvious to a person of ordinary skill in the art, at the time the invention was made, to have modified the methylotrophic Pichia yeast cell, as previously disclosed by Lin-Cereghino, to include a nucleic acid encoding at least one polypeptide involved in heme biosynthesis, as previously disclosed by Nipro, as the previous disclosure by Lin-Cereghino includes a method to improve growth of Pichia yeast cell on a growth medium to enhance the production of recombinant proteins (Lin-Cereghino; abstract; page 883, column 2, paragraph 2). Nipro discloses a heme protein produced in Pichia yeast cells (Nipro, abstract), and a combination of these references would have resulted in a superior methylotrophic Pichia yeast cell for producing a polypeptide involved in heme biosynthesis.

-***-Continued Within the Next Supplemental Box-***-

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US16/31797

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of:

..***-Continued from Box V: Citations and Explanations-***-

As per claim 31, Lin-Cereghino, Impossible, and Nipro, in combination, disclose the yeast cell of claim 30, and Lin-Cereghino further discloses wherein the nucleic acid encoding the Mxr1 transcriptional activator sequence is operably linked to a methanol-inducible promoter element (Mxr1p specifically binds to AOX1 5' promoter sequences (a methanol-inducible promoter); page 884, column 1, paragraph 2).

As per claim 32, Lin-Cereghino, Impossible, and Nipro, in combination, disclose the yeast cell of claim 30, but Lin-Cereghino does not disclose wherein the member of the globin family is leghemoglobin. Impossible discloses wherein the member of the globin family is leghemoglobin (a leghemoglobin protein derived from Pichia pastoris; page 1, paragraph 1). It would have been obvious to a person of ordinary skill in the art, at the time the invention was made, to have modified the methylotrophic Pichia yeast cell, as previously disclosed by Lin-Cereghino, to include wherein the member of the globin family is leghemoglobin, as previously disclosed by Impossible, as the previous disclosure by Lin-Cereghino includes a method to improve growth of Pichia yeast cell on a growth medium to enhance the production of recombinant proteins (Lin-Cereghino; abstract; page 883, column 2, paragraph 2), Impossible discloses a soybean protein produced in Pichia yeast cells (Impossible, page 1, paragraph 1), and a combination of these references would have resulted in a superior methylotrophic Pichia yeast cell for producing a member of the globin family.

As per claims 33/30-32, Lin-Cereghino, Impossible, and Nipro, in combination, disclose the yeast cell of claims 30-32, but Lin-Cereghino does not disclose wherein the nucleic acid encoding the member of the globin family is operably linked to a methanol-inducible promoter element. Impossible discloses wherein the nucleic acid encoding the member of the globin family is operably linked to a methanol-inducible promoter element (the LegH plasmid was integrated into the genome of Bg10 cells at the AOX1 promoter (a methanol-inducible promoter element); page 8, paragraph 2). It would have been obvious to a person of ordinary skill in the art, at the time the invention was made, to have modified the methylotrophic Pichia yeast cell, as previously disclosed by Lin-Cereghino, to include wherein the nucleic acid encoding the member of the globin family is operably linked to a methanol-inducible promoter element, as previously disclosed by Impossible, as the previous disclosure by Lin-Cereghino includes a method to improve growth of Pichia yeast cell on a growth medium to enhance the production of recombinant proteins (Lin-Cereghino; abstract; page 883, column 2, paragraph 2), Impossible discloses a soybean protein produced in Pichia yeast cells (Impossible, page 1, paragraph 1), and a combination of these references would have resulted in a superior methylotrophic Pichia yeast cell for producing a member of the globin family.

As per claims 34/30-32, Lin-Cereghino, Impossible, and Nipro, in combination, disclose the yeast cell of claims 30-32, and Lin-Cereghino further discloses wherein the nucleic acid is operably linked to a constitutive promoter element (the promoter region from the GAP gene (a constitutive promoter) is fused to sequence; page 889, column 1, paragraph 3). Lin-Cereghino does not disclose wherein the nucleic acid encoding the member of the globin family is operably linked. Impossible discloses wherein the nucleic acid encoding the member of the globin family is operably linked (the LegH plasmid was integrated into the genome of Bg10 cells at the AOX1 promoter; page 8, paragraph 2). It would have been obvious to a person of ordinary skill in the art, at the time the invention was made, to have modified the methylotrophic Pichia yeast cell, as previously disclosed by Lin-Cereghino, to include wherein the nucleic acid encoding the member of the globin family is operably linked, as previously disclosed by Impossible, as the previous disclosure by Lin-Cereghino includes a method to improve growth of Pichia yeast cell on a growth medium to enhance the production of recombinant proteins (Lin-Cereghino; abstract; page 883, column 2, paragraph 2), Impossible discloses a soybean protein produced in Pichia yeast cells (Impossible, page 1, paragraph 1), and a combination of these references would have resulted in a superior methylotrophic Pichia yeast cell for producing a member of the globin family.

Claims 1-2, 3/1-2, 4/1-2, 30-32, 33/30-32, and 34/30-32 have industrial applicability as defined by PCT Article 33(4) because the subject matter can be made or used in industry.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING TRANSMITTAL OF COPY OF INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY (CHAPTER I OF THE PATENT COOPERATION TREATY)
(PCT Rule 44bis.1(c))

To:

PARSONS, M. Angela
Fish & Richardson P.C.
P.O. Box 1022
Minneapolis, Minnesota 55440-1022
ETATS-UNIS D'AMERIQUE

Date of mailing *(day/month/year)*	
23 November 2017 (23.11.2017)	
Applicant's or agent's file reference	IMPORTANT NOTICE
387670046WO1	

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US2016/031797	11 May 2016 (11.05.2016)	11 May 2015 (11.05.2015)

Applicant
IMPOSSIBLE FOODS INC.

The International Bureau transmits herewith a copy of the international preliminary report on patentability (Chapter I of the Patent Cooperation Treaty)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

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Authorized officer
Kihwan Moon

e-mail: pct.team1@wipo.int

Electronic Acknowledgement Receipt

EFS ID:	31074580
Application Number:	15678342
International Application Number:	
Confirmation Number:	1018
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Customer Number:	26191
Filer:	M. Angela Parsons/Shannon Stadnick
Filer Authorized By:	M. Angela Parsons
Attorney Docket Number:	38767-0048002
Receipt Date:	29-NOV-2017
Filing Date:	16-AUG-2017
Time Stamp:	13:33:38
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	Transmittal.pdf	69305 711b666522e16b8c8a0eee5ec1461b25c848f2d0	no	1
Warnings:					

Information:					
This is not an USPTO supplied IDS fillable form					
2	Information Disclosure Statement (IDS) Form (SB08)	1449.pdf	119217 5c49d2fb3193710c1691c616939a2bd442c89993	no	1
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
3	Foreign Reference	EP2058398.pdf	485007 62883e0cd2600fab654e1ccd3c3803848b03b6db	no	27
Warnings:					
Information:					
4	Foreign Reference	WO2014008729.pdf	1667092 f9bb075625ee49a2eeaa9a7825b9c637d53488c1	no	34
Warnings:					
Information:					
5	Non Patent Literature	IPRP_PCTUS2016031797_2017_11_14.pdf	425622 52a6b196a2a0a9d60e3d2e64d846d9ba2f4eaf7a	no	7
Warnings:					
Information:					
		Total Files Size (in bytes):	2766243		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Sheet 1 of 1

Substitute Disclosure Form / U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No.	Application No.
Information Disclosure Statement by Applicant (Use several sheets if necessary) (37 CFR §1.98(b))	38767-0048002	15/678,342
	Applicant: Impossible Foods Inc.	
	Filing Date: August 16, 2017	Group Art Unit: 1636

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1.	4,886,753	12/01/1989	Marcker			
	2.	2007/0031832	02/08/2007	Watt			
	3.	2017/0188612	07/06/2017	Varadan			

Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
	4.	WO 2009/009142	01/01/2009	WIPO				

Other Documents (include Author, Title, Date, and Place of Publication)

Examiner Initial	Desig. ID	Document
	5.	Lin-Cereghino et al., "Mxr1p, a key regulator of the methanol utilization pathway and peroxisomal genes in Pichia pastoris," Molecular and Cellular Biology, 26:883-897 (February 2006)
	6.	Sievers et al., "The Primary Structure of Soybean (Glycine max) Leghemoglobin c*," Acta Chemica Scandinavico B, 32:380-386 (1978)

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.S.K/

Examiner Signature	Date Considered
/JAMES S KETTER/	11/29/2017

EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Substitute Disclosure Form

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S1	1	(pichia or pastoris or yeast or yeasts or methylotrophic) same (globin or globins or hemaglobin$ or hemeglobin$ or leghem$) same (ALA adj synthase or ALA adj dehydratase or UPGIII adj synthase or UPGIII adj decarboxylase or CPG adj oxidase or PPG adj oxidase or Porphobilinogen adj deaminase or Uroporphyrinogen adj2 synthase)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2017/09/22 15:43
S2	1	(US-20170188612-$).did.	US-PGPUB	OR	OFF	2017/09/22 15:45
S3	0	mxr1$ and S2	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2017/09/22 15:52
S4	1	leghem$ and S2	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2017/09/22 15:55
S5	1719	(elongation adj factor adj "1" or tef1) same (pichia or pastoris or yeast or yeasts or methylotrophic)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2017/09/22 16:34
S6	1028	(elongation adj factor adj "1" or tef1) with (pichia or pastoris or yeast or yeasts or methylotrophic)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2017/09/22 16:34
S7	216	(elongation adj factor adj "1" or tef1) same (pichia or pastoris or yeast or yeasts or methylotrophic) same (globin or globins or hemaglobin$ or hemeglobin$ or leghem$)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2017/09/22 16:35
S8	175	((elongation adj factor adj "1" or tef1) with (promot$)) same (pichia or pastoris or yeast or yeasts or methylotrophic) same (globin or globins or hemaglobin$ or hemeglobin$ or leghem$)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2017/09/22 16:37
S9	298	((elongation adj factor adj "1" or tef1) with (promot$)) with (pichia or pastoris or methylotrophic)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2017/09/22 17:08
S10	281	((elongation adj factor adj "1" or tef1) adj6 (promot$)) with (pichia or pastoris or methylotrophic)	US-PGPUB; USPAT; EPO; JPO;	OR	OFF	2017/09/22 17:08

EAST Search History

			DERWENT			
S11	170	((elongation adj factor adj "1" or tef1) adj6 (promot$)) with (pichia or pastoris or methylotrophic) with (globin or globins or hemaglobin$ or hemeglobin$ or leghem$)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2017/09/22 17:14
S12	0	(((elongation adj factor adj "1" or tef1) adj6 (promot$)) with (pichia or pastoris or methylotrophic) with (globin or globins or hemaglobin$ or hemeglobin$ or leghem$)) and (heme with (synthetic$ or biosynth$ or synthesi$ or production or produce$ or producing))	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2017/11/29 07:19
S13	8	(((elongation adj factor adj "1" or tef1) adj6 (promot$)) with (pichia or pastoris or methylotrophic) with (globin or globins or hemaglobin$ or hemeglobin$ or leghem$)) and (heme with (gene or genes))	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2017/11/29 07:24

11/29/2017 8:41:21 PM
C:\Users\jketter\Documents\EAST\Workspaces\678342.wsp

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/678,342	08/16/2017	Smita Shankar	38767-0048002	1018

26191 7590 12/04/2017
FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MN 55440-1022

EXAMINER	
KETTER, JAMES S	

ART UNIT	PAPER NUMBER
1636	

NOTIFICATION DATE	DELIVERY MODE
12/04/2017	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

PATDOCTC@fr.com

Office Action Summary	Application No. 15/678,342	Applicant(s) Shankar et al.	
	Examiner JAMES S KETTER	Art Unit 1636	AIA Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____ .

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____ .

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☑ Claim(s) 1-13 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☑ Claim(s) 1,4-7 and 10-13 is/are rejected.

8)☑ Claim(s) 2-3 and 8-9 is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____ .

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date 8/16/17; 10/31/17; 11/29/17 .

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____ .

4) ☐ Other: _____ .

Claims 2, 3, 8 and 9 are objected to as being dependent upon a rejected base claim, but would be allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claims.

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(1) - 706.02(l)(3) for applications not subject to examination under the first inventor

to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

Claims 1-13 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1, 1, 4, 5, 7, 8, 10, 12, 13, 15 and 19-21, respectively, of copending Application No. 15/678,891 (reference application). Although the claims at issue are not identical, they are not patentably distinct from each other because, in each instance, the claim of the instant application encompasses all that is covered by the copending claim, i.e., is generic thereto.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

The following is a quotation of the first paragraph of 35 U.S.C. 112(a):

> (a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of the first paragraph of pre-AIA 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 1, 4-7, 10 and 13 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor or a joint inventor, or for pre-AIA the inventor(s), at the time the application was filed, had possession of the claimed invention.

The MPEP states that the purpose of the written description requirement is to ensure that the inventor had possession, as of the filing date of the application, of the specific subject matter later claimed by him. The courts have stated:

> To fulfill the written description requirement, a patent specification must describe an invention and do so in sufficient detail that one skilled in the art can clearly conclude that "the inventor invented the claimed invention." *Lockwood v. American Airlines, Inc.*, 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997); *In re Gostelli*, 872 F.2d 1008, 1012, 10 USPQ2d 1614, 1618 (Fed. Cir. 1989) ("[T]he description must clearly allow persons of ordinary skill in the art to recognize that [the inventor] invented what is claimed."). Thus, an applicant complies with the written description requirement "by describing the invention, with all its claimed limitations, not that which makes it obvious," and by using "such descriptive means as words, structures, figures, diagrams, formulas, etc., that set forth the claimed invention." *Lockwood*, 107 F.3d at 1572, 41 USPQ2d at 1966." *Regents of the University of California v. Eli Lilly & Co.*, 43 USPQ2d 1398.

Further, for a broad generic claim, the specification must provide adequate written description to identify the genus of the claim. In *Regents of the University of California v. Eli Lilly & Co.* the court stated:

> A written description of an invention involving a chemical genus, like a description of a chemical species, "requires a precise definition, such as by structure, formula, [or] chemical name," of the claimed subject matter sufficient to distinguish it from other materials. *Fiers*, 984 F.2d at 1171, 25 USPQ2d 1601; *In re Smythe*, 480 F.2d 1376, 1383, 178 USPQ 279, 284985 (CCPA 1973) ("In other cases, particularly but not necessarily, chemical cases, where there is unpredictability in performance of certain species or subcombinations other than those specifically enumerated, one skilled in the art may be found not to have been placed in possession of a genus …") *Regents of the University of California v. Eli Lilly & Co.*, 43 USPQ2d 1398.

The MPEP further states that if a biomolecule is described only by a functional characteristic, without any disclosed correlation between function and structure of the sequence, it is "not a sufficient characteristic for written description purposes, even when accompanied by a method of obtaining the claimed sequence." MPEP § 2163. The MPEP does state that for a generic claim the genus can be adequately described if the disclosure presents a sufficient number of representative species that encompass the genus. MPEP § 2163. If the genus has a substantial variance, the disclosure must describe a sufficient variety of species to reflect the variation within that genus. See MPEP § 2163. Although the MPEP does not define what constitute a sufficient number of representative species, the courts have indicated what do not constitute a representative number of species to adequately describe a broad generic. In *Gostelli*, the courts determined that the disclosure of two chemical compounds within a subgenus did not describe that subgenus. *In re Gostelli*, 872, F.2d at 1012, 10 USPQ2d at 1618.

The MPEP lists factors that can be used to determine if sufficient evidence of possession has been furnished in the disclosure of the Application. These include: (1) Actual reduction to practice, (2) Disclosure of drawings or structural chemical formulas, (3) Sufficient relevant identifying characteristics (such as: i. Complete structure, ii. Partial structure, iii. Physical and/or chemical properties, iv. Functional characteristics when coupled with a known or disclosed, and correlation between function and structure), (4) Method of making the claimed invention, (5)

Level of skill and knowledge in the art, and (6) Predictability in the art.

"Disclosure of any combination of such identifying characteristics that distinguish the claimed invention from other materials and would lead one of skill in the art to the conclusion that the applicant was in possession of the claimed species is sufficient." MPEP § 2163. While all of the factors have been considered, a sufficient amount for a *prima facie* case are discussed below.

In the instant case, the claims are drawn to a genus of methylotrophic Pichia yeast cell comprising a nucleic acid molecule encoding a heme-containing protein. However, only the expression of globin is described in the instant specification. No significant description of expression of any other polypeptides which bind heme is set forth. The art does provide a significant sample of successful recombinant expression of heme-binding polypeptides from yeast, particularly from Pichia. As such, one of skill would not have envisioned the full scope of the claimed invention.

The following is a quotation of 35 U.S.C. 112(b):
(b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:
The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 11 and 12 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

The instant claims recite "the leghemoglobin". However, this term lacks antecedent basis in claim 1, from which the instant claims depend.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to JAMES S KETTER whose telephone number is (571)272-0770. The examiner can normally be reached on M-F 9:00-5:30.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Anne Gussow can be reached on 571-272-6047. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would

like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/JAMES S KETTER/
Primary Examiner, Art Unit 1636

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/678,342	Shankar et al.
	Examiner	Art Unit
	JAMES S KETTER	1636

CPC - Searched*		
Symbol	Date	Examiner
No classified search made.	11/29/2017	JK

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
Inventors' names searched.	11/29/2017	JK
Priority checked.	11/29/2017	JK
BRS search attached,	11/29/2017	JK
[MEDLINE search terms and strategy as for BRS search.]	11/29/2017	JK

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

Sheet 1 of 1

Substitute Disclosure Form (37 CFR §1.98(b))	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No.	Application No.
Information Disclosure Statement by Applicant (Use several sheets if necessary)		38767-0048002	15/678,342
		Applicant: Impossible Foods Inc.	
		Filing Date: August 16, 2017	Group Art Unit: 1636

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
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Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
	1.	EP 2058398	05/13/2009	EP				
	2.	WO 2014/008729	01/16/2014	WIPO				

Other Documents (include Author, Title, Date, and Place of Publication)

Examiner Initial	Desig. ID	Document
	3.	International Preliminary Report on Patentability in International Application No. PCT/US2016/031797, dated November 14, 2017, 7 pages

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.S.K/

Examiner Signature	Date Considered
/JAMES S KETTER/	11/29/2017

EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Substitute Disclosure Form

Sheet 1 of 3

Substitute Disclosure Form	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 38767-0048002	Application No. 15/678,342
Information Disclosure Statement by Applicant (Use several sheets if necessary)		Applicant Impossible Foods Inc.	
(37 CFR §1.98(b))		Filing Date August 16, 2017	Group Art Unit

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1.	4,683,195	07/28/1987	Mullis et al.			
	2.	4,683,202	07/28/1987	Mullis			
	3.	4,800,159	01/24/1989	Mullis et al.			
	4.	4,965,188	10/23/1990	Mullis et al.			
	5.	5,204,253	04/20/1993	Sanford et al.			
	6.	5,538,800	07/23/1996	Jin et al.			
	7.	6,013,863	01/11/2000	Lundquist et al.			
	8.	6,329,571	12/11/2001	Hiei			
	9.	2002/00194643	12/19/2002	Bertrand et al.			
	10.	2008/10166757	06/01/2008	Bron et al.			

Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
	11.	WO 1998/012913	04/02/1998	WIPO				
	12.	WO 2004/057946	07/15/2004	WIPO				
	13.	WO 2004/099405	11/18/2004	WIPO				
	14.	WO 2012/083424	06/28/2012	WIPO				
	15.	WO 2013/010042	01/17/2013	WIPO				
	16.	WO 2014/110532	07/17/2014	WIPO				
	17.	WO 2014/110539	07/17/2014	WIPO				

Other Documents (include Author, Title, Date, and Place of Publication)

Examiner Initial	Desig. ID	Document
	18.	Chenna et al., "Multiple sequence alignment with the Clustal series of programs" Nucleic Acids Res., 31(13):3497-500 (2003)
	19.	Chiruvolu et al., "Recombinant protein production in an alcohol oxidase-defective strain of Pichia pastoris in fedbatch fermentations" Enzyme Microb. Technol., 21: 277-83 (1997)

Examiner Signature	Date Considered
/JAMES S KETTER/	11/29/2017

EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Substitute Disclosure Form

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.S.K/

Substitute Disclosure Form	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 38767-0048002	Application No. 15/678,342
Information Disclosure Statement by Applicant (Use several sheets if necessary)		Applicant Impossible Foods Inc.	
(37 CFR §1.98(b))		Filing Date August 16, 2017	Group Art Unit

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
	20.	Dayhoff et al., "A Model of Evolutionary Change in Proteins," Atlas of Protein Sequence and Structure, 5: 345-352 (1978)
	21.	GenBank Accession No. AB365355, "Candida boidinii TRM1 gene for Zn(II)2Cys6-type transcription factor Trm1, complete cds," dated January 24, 2008, 3 pages
	22.	GenBank Accession No. AB548760, "Candida boidinii TRM2 gene for C2H2-type transcription factor Trm2, complete cds," dated May 14, 2010, 3 pages
	23.	GenBank Accession No. ABD57365, "methanol expression regulator I [Komagataella pastoris]," dated March 4, 2006, 2 pages
	24.	GenBank Accession No. AEOI02000005, bases 858873 to 862352, "Ogataea parapolymorpha DL-1 chr3, whole genome shotgun sequence," dated December 3, 2013, 186 pages
	25.	GenBank Accession No. BAF99700, "Zn(II)2Cys6-type transcription factor Trm1 [Candida boidinii]," dated January 23, 2008, 2 pagges
	26.	GenBank Accession No. BAJ07608, "C2H2-type transcription factor Trm2 [Candida boidinii]," dated May 13, 2010, 2 pages
	27.	GenBank Accession No. DQ395124, "Pichia pastoris methanol expression regulator I gene, complete cds," dated March 4, 2006, 2 pages
	28.	GenBank Accession No. ESX01253, "Regulatory protein ADR1 [Ogataea parapolymorpha DL-1]," dated December 2, 2013, 2 pages
	29.	GenBank Accession No. FJ75255l, "Pichia pastoris dihydroxyacetone synthase 1 (DAS1) gene, complete cds" dated March 21, 2009, 2 pages
	30.	GenBank Accession No. X02425, "Hansenula polymorpha MOX gene for methanol oxidase" dated April 20, 1993, 3 pages
	31.	GenBank Accession No. YSAAODlA, "Candida boidinii methanol oxidase (AOD1) gene, complete cds," dated April 27, 1993, 2 pages
	32.	Hargrove et al., "Characterization of recombinant soybean leghemoglobin a and apolar distal histidine mutants" J Mol. Biol. 266, 1032-1042 (1997)
	33.	Inan & Meagher, "Non-repressing carbon sources for alcohol oxidase (AOX1) promoter of Pichia pastoris" J. Biosci. Bioeng., 92:585-9 (2001)
	34.	International Search Report and Written Opinion in International Application No. PCT/US2014/055227, dated March 20, 2015, 20 pages
	35.	Jokipii-Lukkari et al., "Intrinsic non-symbiotic and truncated haemoglobins and heterologous Vitreoscilla haemoglobin expression in plants," Journal of Experimental Botany, 60(2):409-422 (January 2009)
	36.	Kranthi et al., "Identification of Mxr1p-binding sites in the promoters of genes encoding dihydroxyacetone synthase and peroxin 8 of the methylotrophic yeast Pichia pastoris" Yeast, 27:705-11 (2010)
	37.	Liachko & Dunham, "An autonomously replicating sequence for use in a wide range of budding yeasts" FEMS Yeast Res., 14:364-7 (2014)
	38.	Lloyd et al., "Targeted mutagenesis using zinc-finger nucleases in Arabidopsis" Proc. Natl. Acad. Sci. USA, 102:2232-2237 (2005)

Examiner Signature	Date Considered
/JAMES S KETTER/	11/29/2017

EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Substitute Disclosure Form

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Sheet 3 of 3

Substitute Disclosure Form	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 38767-0048002	Application No. 15/678,342
Information Disclosure Statement by Applicant (Use several sheets if necessary)		Applicant Impossible Foods Inc.	
(37 CFR §1.98(b))		Filing Date August 16, 2017	Group Art Unit

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
	39.	Londer et al. "Production and preliminary characterization of a recombiant triheme cytochrom c(7) from Geobatcher sulfurreducens in Escherichia coli." Biochem Biophy Act 1 1554(3): 202-211 ~~(200)~~ (2002).
	40.	Lutz et al., "A guide to choosing vectors for transformation of the plastid genome of higher plants" Plant Physiol. 145: 1201-1210 (2007)
	41.	Miele et al., "A GATA-type transcription factor regulates expression of the high-affinity iron uptake system in the methylotrophic yeast Pichia pastoris" Arch. Biochem. Biophys., 465: 172-9 (2007)
	42.	Nakagawa et al., "Alcohol oxidase hybrid oligomers formed in vivo and in vitro" Yeast, 15: 1223-30 (1999)
	43.	Qu et al., "Ectopic expression of the cottong non-symbiotic hemoglobin gene GhHb1 triggers defense responses and increases disease tolerance in Arabidopsis," Plant Cell Physiol., 47:1058-1068 (2006)
	44.	Raymond et al., "Development of the methylotrophic yeast Pichia methanolica for the expression of the 65 kilodalton isoform of human glutamate decarboxylase" Yeast, 14: 11-23 (1998)
	45.	Richards et al., "Construction of a GFP-BAR plasmid and its use for switchgrass transformation" Plant Cell. Rep. 20:48-20 54 (2001)
	46.	Sinagawa-Garcia et al., "Next generation synthetic vectors for transformation of the plastid genome of higher plants" Plant Afol Biol 70: 487-498 (2009)
	47.	Somleva et al., "Agrobacterium-Mediated Genetic Transformation of Switchgrass" Crop Sci. 42:2080-2087 (2002)
	48.	Supplementary European Search Report in International Application No. EP 14844701.4, dated January 27, 2017, 7 pages
	49.	Tanaka and Tanaka, "Tetrapyrrole biosynthesis in higher plants" Annu. Rev. Plant Biol. 58: 321-46 (2007)
	50.	Tovkach et al., "A toolbox and procedural notes for characterizing novel zinc finger nucleases for genome editing in plant cells" Plant J, 57: 747-757 25 (2009)
	51.	Townsend et al., "High-frequency modification of plant genes using engineered zinc-finger nucleases" Nature 459: 442-445 (2009)
	52.	UniprotKB Accession No. P02236, "Leghemoglobin C2," dated November 1, 1988, 9 pages
	53.	Wu et al., "Efficient production of a functional single-chain antidigoxin antibody via an engineered Bacillus subtilis expression-secretion system" Bio/Technology (NY) 11(1): 71-76 (1993) (abstract only)
	54.	Xie and Yang, "RNA-guided genome editing in plants using a CRISPR-Cas system" Mal. Plant 6: 1975-1983 (2013)
	55.	Zhang et al., "Transcription activator-like effector nucleases enable efficient plant genome engineering" Plant Physiology 161: 20-27 (2013)

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.S.K/

Examiner Signature	Date Considered
/JAMES S KETTER/	11/29/2017

EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Substitute Disclosure Form

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/678,342	08/16/2017	Smita Shankar	38767-0048002

CONFIRMATION NO. 1018

26191
FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MN 55440-1022

PUBLICATION NOTICE

OC000000095939420

Title:EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST

Publication No.US-2017-0349906-A1
Publication Date:12/07/2017

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Public Records Division. The Public Records Division can be reached by telephone at (571) 272-3150 or (800) 972-6382, by facsimile at (571) 273-3250, by mail addressed to the United States Patent and Trademark Office, Public Records Division, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently https://portal.uspto.gov/pair/PublicPair. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

Attorney Docket No.: 38767-0048002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Smita Shankar	Art Unit	:	1636
Serial No.	:	15/678,342	Examiner	:	James S. Ketter
Filed	:	August 16, 2017	Conf. No.	:	1018
Title	:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST			

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Please consider the reference listed on the enclosed PTO-1449 Form. A non-patent literature reference is enclosed.

This statement is being filed after a first Office Action on the merits, but before receipt of a Final Office Action or a Notice of Allowance. A submission fee in the amount of $240, as required under 37 CFR § 1.17(p), is being paid with this statement. Please apply any other charges, or credits, to Deposit Account No. 06-1050, referencing the above-indicated Attorney Docket Number.

Please contact the undersigned if there are any questions regarding this statement.

Respectfully submitted,

Date: /March 1, 2018/

/M. Angela Parsons/
M. Angela Parsons, Ph.D.
Reg. No. 44,282

Customer Number 26191
Fish & Richardson P.C.
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61639765.doc

Sheet 1 of 1

Substitute Disclosure Form (37 CFR §1.98(b))	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 38767-0048002	Application No. 15/678,342
Information Disclosure Statement by Applicant (Use several sheets if necessary)		Applicant Impossible Foods Inc.	
		Filing Date August 16, 2017	Group Art Unit 1636

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	--	--	--	--			

Foreign Patent Documents or Published Foreign Patent Applications								
Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation	
							Yes	No
	--	--	--	--				

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
	1.	Cregg et al, "Recombinant protein expression in Pichia pastoris," Molecular Biotechnology, 16:23-52 (2000)

Examiner Signature	Date Considered

EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Substitute Disclosure Form

Electronic Patent Application Fee Transmittal

Application Number:	15678342
Filing Date:	16-Aug-2017
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Filer:	M. Angela Parsons/Jessica Ruettimann
Attorney Docket Number:	38767-0048002

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Submission- Information Disclosure Stmt	1806	1	240	240
		Total in USD ($)		**240**

Electronic Acknowledgement Receipt

EFS ID:	31928310
Application Number:	15678342
International Application Number:	
Confirmation Number:	1018
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Customer Number:	26191
Filer:	M. Angela Parsons/Jessica Ruettimann
Filer Authorized By:	M. Angela Parsons
Attorney Docket Number:	38767-0048002
Receipt Date:	01-MAR-2018
Filing Date:	16-AUG-2017
Time Stamp:	10:27:00
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$240
RAM confirmation Number	030118INTEFSW00013873061050
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	IDS_Transmittal.pdf	72231 44345ef199c34e0f0bf80a5bcd08e98540fd36e7	no	1
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
2	Information Disclosure Statement (IDS) Form (SB08)	PTO_1449.pdf	122852 1081aa20e11296d13ce21797ddd802bb2710076b	no	1
Warnings:					
Information:					
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3	Non Patent Literature	Cregg_1.pdf	12625710 551eb50069a616eca2efe326d29877ebca999161	no	15
Warnings:					
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4	Non Patent Literature	Cregg_2.pdf	16941012 7a2bae1a0be77d90e07674bb08c830c5b1da66a0	no	15
Warnings:					
Information:					
5	Fee Worksheet (SB06)	fee-info.pdf	30683 60d7531889f5ebc9a7812e2e8f9dc69cf7a68337	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	29792488		

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Attorney Docket No.: 38767-0048002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Smita Shankar	Art Unit	:	1636
Serial No.	:	15/678,342	Examiner	:	James S. Ketter
Filed	:	August 16, 2017	Conf. No.	:	1018
Title	:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST			

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

RESPONSE TO OFFICE ACTION MAILED DECEMBER 4, 2017

In response to the Office Action mailed December 4, 2017, please consider the following reply.

Amendments to the Claims:

Please amend claims 11 and 12 and add new claim 14 as follows. The claims and their status are shown below.

1. (Original) A methylotrophic *Pichia* yeast cell comprising:

a nucleic acid molecule encoding a heme-containing protein operably linked to a promoter element from *P. pastoris* and a Mxr1 transcriptional activator sequence from *P. pastoris*; and

a nucleic acid molecule encoding at least one polypeptide involved in heme biosynthesis operably linked to a promoter element from *P. pastoris* and a Mxr1 transcriptional activator sequence from *P. pastoris*.

2. (Original) The yeast cell of claim 1, wherein the heme-containing protein is Leghemoglobin (LegH).

3. (Original) The yeast cell of claim 2, wherein the LegH has the sequence shown in SEQ ID NO:6.

4. (Original) The yeast cell of claim 1, wherein the at least one polypeptide involved in heme biosynthesis is selected from the group consisting of ALA synthase, ALA dehydratase, porphogilinogen deaminase, UPG III synthase, UPG III decarboxylase, CPG oxidase, PPG oxidase, and/or ferrochelatase

5. (Original) The yeast cell of claim 1, wherein the promoter element from *P. pastoris* is a constitutive promoter element from *P. pastoris* or a methanol-inducible promoter element from *P. pastoris*.

6. (Original) The yeast cell of claim 5, wherein the constitutive promoter element from *P. pastoris* is a promoter element from a transcriptional elongation factor EF-1 (TEF1) gene.

7. (Original) The yeast cell of claim 5, wherein the methanol-inducible promoter element from *P. pastoris* is an alcohol oxidase I (AOX1) promoter element.

8. (Original) The yeast cell of claim 1, wherein the promoter element from *P. pastoris* comprises a constitutive promoter element from *P. pastoris* and a methanol-inducible promoter element from *P. pastoris*.

9. (Original) The yeast cell of claim 8, wherein the constitutive promoter element from *P. pastoris* is a promoter element from a transcriptional elongation factor EF-1 (TEF1) gene.

10. (Original) The yeast cell of claim 8, wherein the methanol-inducible promoter element from *P. pastoris* is an alcohol oxidase I (AOX1) promoter element.

11. (Currently Amended) The yeast cell of claim 1, wherein the nucleic acid molecule encoding the heme-containing protein ~~leghemoglobin~~ is present in the yeast cell in at least two copies.

12. (Currently Amended) The yeast cell of claim 1, wherein the nucleic acid molecule encoding the heme-containing protein ~~leghemoglobin~~ operably linked to the promoter element from *P. pastoris* and the Mxr1 transcriptional activator sequence from *P. pastoris* further comprises a termination sequence.

13. (Original) The yeast cell of claim 1, wherein the nucleic acid encoding the at least one polypeptide involved in heme biosynthesis operably linked to the promoter element from *P. pastoris* and the Mxr1 transcriptional activator sequence from *P. pastoris* further comprises a termination sequence.

14. (New) The yeast cell of claim 1, further comprising a nucleic acid molecule encoding a Mxr1 transcriptional activator operably linked to a promoter element from *P. pastoris* and a Mxr1 transcriptional activator sequence from *P. pastoris*.

REMARKS

Applicants respectfully request entry of the amendments and remarks submitted herein. Claims 11 and 12 have been amended herein as discussed in more detail below, and new claim 14 has been added. Support for new claim 14 can be found, for example, in the pMx354 vector shown in Figure 4.

Claims 1-14 are currently pending. Applicants thank the Examiner for indicating that claims 2, 3, 8 and 9 are allowable. Reconsideration of the pending application is respectfully requested.

The Double Patenting Rejection

Claims 1-13 are provisionally rejected on the ground of non-statutory double patenting as being unpatentable over "claims 1, 1, 4, 5, 7, 8, 10, 12, 13, 15 and 19-21" of co-pending U.S. Application No. 15/678,891 (OA at page 3; given the duplication of the "1" in the listing of the claims above, Applicants respectfully request clarification of the claims in U.S. Application No. 15/678,891 over which the present claims are rejected).

Applicants respectfully request that this rejection be held in abeyance until allowable subject matter is found. At that time, Applicants will consider submitting an appropriate Terminal Disclaimer.

The 35 U.S.C. § 112 Rejection

Claims 1, 4-7, 10 and 13 are rejected under 35 U.S.C. § 112(a) or 35 U.S.C. § 112 (pre-AIA), first paragraph, as the Examiner asserted that those claims fail to comply with the written description requirement. According to the Examiner, "the claims are drawn to a genus of methylotrophic Pichia yeast cell comprising a nucleic acid molecule encoding a heme-containing protein. However, only the expression of globin is described in the instant specification. No significant description of expression of any other polypeptides which bind heme is set forth" (OA at page 6). Applicants respectfully traverse this rejection as follows.

Contrary to the Examiner's assertion, the instant application refers to "heme containing protein" (see page 7, lines 28-29) and describes members of the globin family of proteins as being in the Pfam family of PF00042 (see page 8, lines 3-5). The instant application also contains multiple references to "leghemoglobin," "LegH" and "heme-bound LegH" (see, for example, page 8, lines 5-11; page 10, lines 12-14; page 10, line 16; page 15, lines 24-28; and throughout the Examples (e.g., Example 19, 20, 22 and 24), and SEQ ID NOs: 3, 4, 5, and 6).

As set forth herein, the instant application provides written description support for the claimed "heme containing protein." Therefore, the pending claims meet the written description requirement, and Applicants respectfully request that the rejection of the pending claims under 35 U.S.C. § 112(a) or 35 U.S.C. § 112 (pre-AIA), first paragraph, be withdrawn.

Claims 11 and 12 are rejected under 35 U.S.C. § 112(b) or 35 U.S.C. § 112 (pre-AIA), second paragraph, as the Examiner asserted that those claims are indefinite. According to the Examiner, these claims lack antecedent basis for "the leghemoglobin" (OA at page 7).

Without acquiescing to the Examiner's rejection, Applicants have amended claims 11 and 12 to replace "leghemoglobin" with "heme-containing protein." Claims 11 and 12, which depend from claim 1, have antecedent basis.

In view of the amendments and remarks herein, Applicants respectfully request that the rejection of claims 11 and 12 under 35 U.S.C. § 112(b) or 35 U.S.C. § 112 (pre-AIA), second paragraph, be withdrawn.

CONCLUSION

Applicants respectfully request that claims 1-14 be allowed. If a telephone call to the undersigned would expedite prosecution, the Examiner is encouraged to do so.

This Response to Office Action is being filed within the three-month Shortened Statutory Period for reply. Therefore, no fees are believed due. If this is incorrect, please apply any charges, or credits, to Deposit Account No. 06-1050, referencing the above-indicated Attorney Docket Number.

Respectfully submitted,

Date: /March 1, 2018/

/M. Angela Parsons/

M. Angela Parsons
Reg. No. 44,282

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EFS ID:	31930401
Application Number:	15678342
International Application Number:	
Confirmation Number:	1018
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Customer Number:	26191
Filer:	M. Angela Parsons/Judith Witzel
Filer Authorized By:	M. Angela Parsons
Attorney Docket Number:	38767-0048002
Receipt Date:	01-MAR-2018
Filing Date:	16-AUG-2017
Time Stamp:	12:29:30
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		Response.pdf	102027 dfde1d3a6490881c8783f9c1c7efc520a06a9ea1	yes	7

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Amendment/Req. Reconsideration-After Non-Final Reject	1	1
	Claims	2	4
	Applicant Arguments/Remarks Made in an Amendment	5	7
Warnings:			
Information:			
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New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.
National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 15/678,342	Filing Date 08/16/2017	☐ To be Mailed

ENTITY: ☒ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)		
FOR	NUMBER FILED	NUMBER EXTRA	RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT	03/01/2018	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 14	Minus	** 20	= 0	X $100 =	0
	Independent (37 CFR 1.16(h))	* 1	Minus	***3	= 0	X $460 =	0
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	0

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

SLIE
MARIDOL GARCIA JOY

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**
If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/678,342	08/16/2017	Smita Shankar	38767-0048002	1018

26191 7590 06/13/2018
FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MINNESOTA 55440-1022
UNITED STATES OF AMERICA

EXAMINER
KETTER, JAMES S

ART UNIT	PAPER NUMBER
1636	

NOTIFICATION DATE	DELIVERY MODE
06/13/2018	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

PATDOCTC@fr.com

PTOL-90A (Rev. 04/07)

Office Action Summary	**Application No.** 15/678,342	**Applicant(s)** Shankar et al.	
	Examiner JAMES S KETTER	**Art Unit** 1636	**AIA Status** Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 1 March 2018.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☑ This action is **FINAL.** 2b) ☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 1-14 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 1-14 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date 3/1/2018.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

Notice of Pre-AIA or AIA Status

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., In re Berg, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); In re Goodman, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); In reLongi, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); In re Van Omum, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); In re Vogel, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); In re Thorington, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(1)(1) - 706.02(1)(3) for applications not subject to examination under the

first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

Claims 1-13 stand, and newly added 14 is, rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1, 4, 5, 7, 8, 10, 12, 13, 15 and 19-21, of US Patent 9,938,327, for reasons set forth in the previous Office Action. (The application referenced in the previous Office Action has now issued as this patent.) Although the claims at issue are not identical, they are not patentably distinct from each other because for reasons of record set forth in the previous Office Action. No response on the merits has been presented, and as such, the rejection must stand.

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after

the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to JAMES S KETTER whose telephone number is (571)272-0770. The examiner can normally be reached on M-F 9:00-5:30.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Anne Gussow can be reached on 571-272-6047. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR

system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/JAMES S KETTER/
Primary Examiner, Art Unit 1636

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/678,342	Shankar et al.
	Examiner	Art Unit
	JAMES S KETTER	1636

CPC - Searched*		
Symbol	Date	Examiner
No classified search made.	11/29/2017	JK
No classified search made.	06/08/2018	JK

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
Inventors' names searched.	11/29/2017	JK
Priority checked.	11/29/2017	JK
BRS search attached,	11/29/2017	JK
[MEDLINE search terms and strategy as for BRS search.]	11/29/2017	JK
Updated from previous searches.	06/08/2018	JK

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

/JAMES S KETTER/ Primary Examiner, Art Unit 1636	

Sheet 1 of 1

Substitute Disclosure Form (37 CFR §1.98(b))	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 38767-0048002	Application No. 15/678,342
Information Disclosure Statement by Applicant (Use several sheets if necessary)		Applicant Impossible Foods Inc.	
		Filing Date August 16, 2017	Group Art Unit 1636

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	--	--	--	--			

Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
	--	--	--	--				

Other Documents (include Author, Title, Date, and Place of Publication)

Examiner Initial	Desig. ID	Document
	1.	Cregg et al, "Recombinant protein expression in Pichia pastoris," Molecular Biotechnology, 16:23-52 (2000)

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.S.K/

Examiner Signature	Date Considered
/JAMES S KETTER/	06/08/2018

EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Substitute Disclosure Form

Attorney Docket No.: 38767-0048002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Smita Shankar	Art Unit	:	1636
Application No.	:	15/678,342	Examiner	:	James S. Ketter
Filed	:	August 16, 2017	Conf. No.	:	1018
Title	:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST			

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Please consider the references listed on the enclosed Form PTO-1449. Foreign patent documents and non-patent literature are enclosed; U.S. patents and patent application publications will be provided upon request.

Applicant also draws the Examiner's attention to the following application. Applicant requests the Examiner review the file history of this application, including the pending claims, which the Examiner can readily access on the USPTO's PAIR website.

Application No.	Filing Date	Inventor	Status
15/573,300	November 10, 2017	Shankar et al.	Published

This statement is being filed after a Final Office Action but before receipt of a Notice of Allowance. A fee in the amount of $240 as required under 37 CFR § 1.17(p) is being paid concurrently with this statement. Please apply any other charges, or credits, to Deposit Account No. 06-1050, referencing the above-indicated Attorney Docket Number.

Please contact the undersigned if there are any questions regarding this statement.

Respectfully submitted,

Date: /June 19, 2018/

/M. Angela Parsons/
M. Angela Parsons, Ph.D.
Reg. No. 44,282

Customer Number 26191
Fish & Richardson P.C.
Telephone: (612) 335-5070
Facsimile: (877) 769-7945
61702808.doc

Sheet 1 of 1

Substitute Disclosure Form (37 CFR §1.98(b))	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No.	Application No.
Information Disclosure Statement by Applicant (Use several sheets if necessary)		38767-0048002	15/678,342
		Applicant: Impossible Foods Inc.	
		Filing Date: August 16, 2017	Group Art Unit: 1636

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1.	2018/0127764	05/10/2018	Shankar et al.			

Foreign Patent Documents or Published Foreign Patent Applications								
Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
	--	--	--	--				

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
	--	--

Examiner Signature	Date Considered

EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Substitute Disclosure Form

Electronic Patent Application Fee Transmittal

Application Number:	15678342
Filing Date:	16-Aug-2017
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Filer:	M. Angela Parsons/Shannon Stadnick
Attorney Docket Number:	38767-0048002

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
SUBMISSION- INFORMATION DISCLOSURE STMT	1806	1	240	240
		Total in USD ($)		**240**

Electronic Acknowledgement Receipt

EFS ID:	32934576
Application Number:	15678342
International Application Number:	
Confirmation Number:	1018
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Customer Number:	26191
Filer:	M. Angela Parsons/Shannon Stadnick
Filer Authorized By:	M. Angela Parsons
Attorney Docket Number:	38767-0048002
Receipt Date:	19-JUN-2018
Filing Date:	16-AUG-2017
Time Stamp:	11:58:30
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$240
RAM confirmation Number	061918INTEFSW00010043061050
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	Transmittal.pdf	92368 d481027774c45ba24f79f24c9354cfb70d508a9a	no	1
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
2	Information Disclosure Statement (IDS) Form (SB08)	1449.pdf	120356 2d882210e259fef2ee2141abd1d13c86c0e51ce0	no	1
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
3	Fee Worksheet (SB06)	fee-info.pdf	30587 4cda9ee9898165350c51914a9e715a5545848a47	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	243311		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.
National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Attorney Docket No.: 38767-0048002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Smita Shankar	Art Unit	:	1636
Application No.	:	15/678,342	Examiner	:	James S. Ketter
Filed	:	August 16, 2017	Conf. No.	:	1018
Title	:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST			

MAIL STOP AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Please consider the reference listed on the enclosed PTO-1449 Form. Non-patent literature is enclosed.

Certification under 37 CFR § 1.97(e)(1)

Each item of information contained in this Information Disclosure Statement was first cited in a Communication from a foreign Patent Office in a counterpart foreign application not more than three months prior to the filing of this Information Disclosure Statement. A submission fee in the amount of $240, as required under 37 CFR § 1.17(p), is being paid with this statement.

Certification under 37 CFR § 1.704(d)

Each item of information contained in this Information Disclosure Statement (i) was first cited in a Communication from a Patent Office in a counterpart foreign or international application or from the Office, and this Communication was not received by an individual designated in 37 CFR § 1.56(c) more than thirty days prior to the filing of the Information Disclosure Statement; or (ii) is a Communication that was issued by a Patent Office in a counterpart foreign or international application or by the Office, and this Communication was not received by an individual designated in § 1.56(c) more than thirty days prior to the filing of the Information Disclosure Statement. Accordingly, no reduction in Patent Term Adjustment (PTA) is warranted by the filing of this Information Disclosure Statement.

First Named Inventor	:	Smita Shankar	Attorney Docket: 38767-0048002
Application No.	:	15/678,342	
Filed	:	August 16, 2017	
Page	:	2 of 2	

This statement is being filed after a Final Office Action or a Notice of Allowance, but before payment of the Issue Fee. Please apply all necessary charges, or credits, to Deposit Account No. 06-1050, referencing the above-indicated Attorney Docket Number.

Please contact the undersigned if there are any questions regarding this statement.

Respectfully submitted,

Date: /July 6, 2018/ /M. Angela Parsons/

M. Angela Parsons, Ph.D.
Reg. No. 44,282

Customer Number 26191
Fish & Richardson P.C.
Telephone: (612) 335-5070
Facsimile: (877) 769-7945

61713406.doc

Sheet 1 of 1

Substitute Disclosure Form (37 CFR §1.98(b))	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No.	Application No.
Information Disclosure Statement by Applicant (Use several sheets if necessary)		38767-0048002	15/678,342
		Applicant: Impossible Foods Inc.	
		Filing Date: August 16, 2017	Group Art Unit: 1636

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	--	--	--	--			

Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
	--	--	--	--				

Other Documents (include Author, Title, Date, and Place of Publication)

Examiner Initial	Desig. ID	Document
	1.	[No Author Listed] Impossible Foods Inc. "GRAS Notification for Soybean Leghemoglobin Protein Derived from Pichia Pastoris." GRAS notice 000737, Retrieved from internet <URL:https://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm, 1063 pages

Examiner Signature	Date Considered

EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Substitute Disclosure Form

Electronic Patent Application Fee Transmittal

Application Number:	15678342
Filing Date:	16-Aug-2017
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Filer:	M. Angela Parsons/Shannon Stadnick
Attorney Docket Number:	38767-0048002

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
SUBMISSION- INFORMATION DISCLOSURE STMT	1806	1	240	240
		Total in USD ($)		**240**

Electronic Acknowledgement Receipt

EFS ID:	33104785
Application Number:	15678342
International Application Number:	
Confirmation Number:	1018
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Customer Number:	26191
Filer:	M. Angela Parsons/Shannon Stadnick
Filer Authorized By:	M. Angela Parsons
Attorney Docket Number:	38767-0048002
Receipt Date:	06-JUL-2018
Filing Date:	16-AUG-2017
Time Stamp:	11:27:55
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$240
RAM confirmation Number	070618INTEFSW00009962061050
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	Transmittal.pdf	73815 ba31801d42b98ae6d03aa3c1c8538487dfaba3c0	no	2
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
2	Information Disclosure Statement (IDS) Form (SB08)	1449.pdf	123070 2a87827f3f6278e95c3b46484cf6820e5a98fc8d	no	1
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
3	Non Patent Literature	GRAS_Notification_Soybean_Leghemoglobin.pdf	16322875 0be2cfd827f92640e30e9c0c934f712b5526b01e	no	1063
Warnings:					
Information:					
4	Fee Worksheet (SB06)	fee-info.pdf	30587 0a73f72daf87df0d94ddffa999fcacef53f71599	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	16550347		

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc Code: DIST.E.FILE **Document Description: Electronic Terminal Disclaimer - Filed**	PTO/SB/26 U.S. Patent and Trademark Office Department of Commerce

Electronic Petition Request	**TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT**
Application Number	15678342
Filing Date	16-Aug-2017
First Named Inventor	Smita Shankar
Attorney Docket Number	38767-0048002
Title of Invention	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST

☒ Filing of terminal disclaimer does not obviate requirement for response under 37 CFR 1.111 to outstanding Office Action

☒ This electronic Terminal Disclaimer is not being used for a Joint Research Agreement.

Owner	Percent Interest
Impossible Foods Inc.	100%

The owner(s) with percent interest listed above in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of prior patent number(s)

9938327

as the term of said prior patent is presently shortened by any terminal disclaimer. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the prior patent are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the owner does not disclaim the terminal part of the term of any patent granted on the instant application that would extend to the expiration date of the full statutory term of the prior patent, "as the term of said prior patent is presently shortened by any terminal disclaimer," in the event that said prior patent later:
- expires for failure to pay a maintenance fee;
- is held unenforceable;
- is found invalid by a court of competent jurisdiction;
- is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321;
- has all claims canceled by a reexamination certificate;
- is reissued; or
- is in any manner terminated prior to the expiration of its full statutory term as presently shortened by any terminal disclaimer.

◉ Terminal disclaimer fee under 37 CFR 1.20(d) is included with Electronic Terminal Disclaimer request.

○ I certify, in accordance with 37 CFR 1.4(d)(4), that the terminal disclaimer fee under 37 CFR 1.20(d) required for this terminal disclaimer has already been paid in the above-identified application.
Applicant claims the following fee status: ○ Small Entity ○ Micro Entity ◉ Regular Undiscounted I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.
THIS PORTION MUST BE COMPLETED BY THE SIGNATORY OR SIGNATORIES I certify, in accordance with 37 CFR 1.4(d)(4) that I am: ◉ An attorney or agent registered to practice before the Patent and Trademark Office who is of record in this application Registration Number 44282 ○ A sole inventor ○ A joint inventor; I certify that I am authorized to sign this submission on behalf of all of the inventors as evidenced by the power of attorney in the application ○ A joint inventor; all of whom are signing this request

Signature	/M. Angela Parsons/
Name	M Angela Parsons

*Statement under 37 CFR 3.73(b) is required if terminal disclaimer is signed by the assignee (owner).
Form PTO/SB/96 may be used for making this certification. See MPEP § 324.

Electronic Patent Application Fee Transmittal

Application Number:	15678342
Filing Date:	16-Aug-2017
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Filer:	M. Angela Parsons/Judith Witzel
Attorney Docket Number:	38767-0048002

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
STATUTORY OR TERMINAL DISCLAIMER	1814	1	160	160
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**160**

Doc Code: DISQ.E.FILE
Document Description: Electronic Terminal Disclaimer – Approved

Application No.: 15678342

Filing Date: 16-Aug-2017

Applicant/Patent under Reexamination: Shankar

Electronic Terminal Disclaimer filed on August 7, 2018

☒ APPROVED

This patent is subject to a terminal disclaimer

☐ DISAPPROVED

Approved/Disapproved by: Electronic Terminal Disclaimer automatically approved by EFS-Web

U.S. Patent and Trademark Office

Electronic Acknowledgement Receipt

EFS ID:	33394167
Application Number:	15678342
International Application Number:	
Confirmation Number:	1018
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Customer Number:	26191
Filer:	M. Angela Parsons/Judith Witzel
Filer Authorized By:	M. Angela Parsons
Attorney Docket Number:	38767-0048002
Receipt Date:	07-AUG-2018
Filing Date:	16-AUG-2017
Time Stamp:	11:50:34
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$160
RAM confirmation Number	080718INTEFSW00010045061050
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Terminal Disclaimer-Filed (Electronic)	eTerminal-Disclaimer.pdf	33472 71fcecd000c4756ffee896fc90fa501dee9e5a3b	no	2
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30593 c76d3ce8d8c03f8e457067892f4b32731e203c35	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	64065		

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New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.
National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Attorney Docket No.: 38767-0048002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Smita Shankar	Art Unit	:	1636
Serial No.	:	15/678,342	Examiner	:	James S. Ketter
Filed	:	August 16, 2017	Conf. No.	:	1018

Title : EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST

MAIL STOP AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

RESPONSE TO OFFICE ACTION MAILED JUNE 13, 2018

In response to the Office Action mailed June 13, 2018, please consider the following reply.

In the Claims

No claims were amended herein. The claims and their status are shown below.

1. (Original) A methylotrophic *Pichia* yeast cell comprising:

a nucleic acid molecule encoding a heme-containing protein operably linked to a promoter element from *P. pastoris* and a Mxr1 transcriptional activator sequence from *P. pastoris*; and

a nucleic acid molecule encoding at least one polypeptide involved in heme biosynthesis operably linked to a promoter element from *P. pastoris* and a Mxr1 transcriptional activator sequence from *P. pastoris*.

2. (Original) The yeast cell of claim 1, wherein the heme-containing protein is Leghemoglobin (LegH).

3. (Original) The yeast cell of claim 2, wherein the LegH has the sequence shown in SEQ ID NO:6.

4. (Original) The yeast cell of claim 1, wherein the at least one polypeptide involved in heme biosynthesis is selected from the group consisting of ALA synthase, ALA dehydratase, porphogilinogen deaminase, UPG III synthase, UPG III decarboxylase, CPG oxidase, PPG oxidase, and/or ferrochelatase

5. (Original) The yeast cell of claim 1, wherein the promoter element from *P. pastoris* is a constitutive promoter element from *P. pastoris* or a methanol-inducible promoter element from *P. pastoris*.

6. (Original) The yeast cell of claim 5, wherein the constitutive promoter element from *P. pastoris* is a promoter element from a transcriptional elongation factor EF-1 (TEF1) gene.

7. (Original) The yeast cell of claim 5, wherein the methanol-inducible promoter element from *P. pastoris* is an alcohol oxidase I (AOX1) promoter element.

8. (Original) The yeast cell of claim 1, wherein the promoter element from *P. pastoris* comprises a constitutive promoter element from *P. pastoris* and a methanol-inducible promoter element from *P. pastoris*.

9. (Original) The yeast cell of claim 8, wherein the constitutive promoter element from *P. pastoris* is a promoter element from a transcriptional elongation factor EF-1 (TEF1) gene.

10. (Original) The yeast cell of claim 8, wherein the methanol-inducible promoter element from *P. pastoris* is an alcohol oxidase I (AOX1) promoter element.

11. (Previously Presented) The yeast cell of claim 1, wherein the nucleic acid molecule encoding the heme-containing protein is present in the yeast cell in at least two copies.

12. (Previously Presented) The yeast cell of claim 1, wherein the nucleic acid molecule encoding the heme-containing protein operably linked to the promoter element from *P. pastoris* and the Mxr1 transcriptional activator sequence from *P. pastoris* further comprises a termination sequence.

13. (Original) The yeast cell of claim 1, wherein the nucleic acid encoding the at least one polypeptide involved in heme biosynthesis operably linked to the promoter element from *P. pastoris* and the Mxr1 transcriptional activator sequence from *P. pastoris* further comprises a termination sequence.

14. (Previously Presented) The yeast cell of claim 1, further comprising a nucleic acid molecule encoding a Mxr1 transcriptional activator operably linked to a promoter element from *P. pastoris* and a Mxr1 transcriptional activator sequence from *P. pastoris*.

REMARKS

Applicants respectfully request entry of the remarks submitted herein. Claims 1-14 are currently pending. Reconsideration of the pending application is respectfully requested.

Non-Statutory Double Patenting Rejection

Claims 1-13 stand, and newly added 14 is, rejected on the ground of non-statutory double patenting as being unpatentable over claims 1, 4, 5, 7, 8, 10, 12, 13, 15 and 19-21, of US Patent 9,938,327, for reasons set forth in the previous Office Action.

Applicants submit herewith an e-Terminal Disclaimer to terminally disclaim over the indicated issued patent. Applicants respectfully request that the non-statutory double patenting rejection be withdrawn.

CONCLUSION

Applicants respectfully request that claims 1-14 be allowed. If a telephone call to the undersigned would expedite prosecution, the Examiner is encouraged to do so.

This Response is being filed within the two month Shortened Statutory Period for reply, and the fee for the Terminal Disclaimer is being paid electronically. Please apply any additional charges, or credits, to Deposit Account No. 06-1050, referencing the above-indicated Attorney Docket Number.

Respectfully submitted,

Date: /August 7, 2018/

/M. Angela Parsons/
M. Angela Parsons, Ph.D.
Reg. No. 44,282

Customer Number 26191
Fish & Richardson P.C.
Telephone: (612) 335-5070
Facsimile: (877) 769-7945

61712280.doc

Electronic Acknowledgement Receipt

EFS ID:	33394363
Application Number:	15678342
International Application Number:	
Confirmation Number:	1018
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Customer Number:	26191
Filer:	M. Angela Parsons/Judith Witzel
Filer Authorized By:	M. Angela Parsons
Attorney Docket Number:	38767-0048002
Receipt Date:	07-AUG-2018
Filing Date:	16-AUG-2017
Time Stamp:	11:57:10
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		RESPNFOA.pdf	95609 c560a6f72fdf8635f8bcc6496e52ae2d7c003760	yes	5

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Response After Final Action	1	1
	Claims	2	4
	Applicant Arguments/Remarks Made in an Amendment	5	5
Warnings:			
Information:			
	Total Files Size (in bytes):	95609	

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New Applications Under 35 U.S.C. 111
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National Stage of an International Application under 35 U.S.C. 371
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New International Application Filed with the USPTO as a Receiving Office
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PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 15/678,342	Filing Date 08/16/2017	☐ To be Mailed

ENTITY: ☒ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)		
FOR	NUMBER FILED	NUMBER EXTRA	RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT	08/07/2018	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 14	Minus	** 20	= 0	X $100 =	0
	Independent (37 CFR 1.16(h))	* 1	Minus	***3	= 0	X $460 =	0
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	0

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

LIE
William Phillips

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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NOTICE OF ALLOWANCE AND FEE(S) DUE

26191 7590 08/15/2018

FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MINNESOTA 55440-1022
UNITED STATES OF AMERICA

EXAMINER
KETTER, JAMES S

ART UNIT	PAPER NUMBER
1636	

DATE MAILED: 08/15/2018

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/678,342	08/16/2017	Smita Shankar	38767-0048002	1018

TITLE OF INVENTION: EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1000	$0.00	$0.00	$1000	11/15/2018

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

26191 7590 08/15/2018
FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MINNESOTA 55440-1022
UNITED STATES OF AMERICA

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/678,342	08/16/2017	Smita Shankar	38767-0048002	1018

TITLE OF INVENTION: EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1000	$0.00	$0.00	$1000	11/15/2018

EXAMINER	ART UNIT	CLASS-SUBCLASS
KETTER, JAMES S	1636	435-254230

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____
2 _____
3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. The following fee(s) are submitted:

❑ Issue Fee

❑ Publication Fee (No small entity discount permitted)

❑ Advance Order - # of Copies _____

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**

❑ A check is enclosed.

❑ Payment by credit card. Form PTO-2038 is attached.

❑ The director is hereby authorized to charge the required fee(s), any deficiency, or credits any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)

❑ Applicant certifying micro entity status. See 37 CFR 1.29

❑ Applicant asserting small entity status. See 37 CFR 1.27

❑ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/678,342	08/16/2017	Smita Shankar	38767-0048002	1018

26191 7590 08/15/2018

FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MINNESOTA 55440-1022
UNITED STATES OF AMERICA

EXAMINER	
KETTER, JAMES S	
ART UNIT	**PAPER NUMBER**
1636	

DATE MAILED: 08/15/2018

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No. 15/678,342	Applicant(s) Shankar et al.	
	Examiner JAMES S KETTER	Art Unit 1636	AIA Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☑ This communication is responsive to the response filed 7 August 2018.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☑ The allowed claim(s) is/are 1-14 . As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see **http://www.uspto.gov/patents/init_events/pph/index.jsp** or send an inquiry to **PPHfeedback@uspto.gov.**

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   Certified copies:

   a) ☐All b) ☐ Some *c) ☐ None of the:

   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file areply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☑ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date 2 X 6/19/2018; 7/6/2018.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date. _____.
5. ☐ Examiner's Amendment/Comment
6. ☐ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/JAMES S KETTER/
Primary Examiner, Art Unit 1636

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/678,342	Shankar et al.
	Examiner	**Art Unit**
	JAMES S KETTER	1636

CPC								
Symbol							**Type**	**Version**
C12N	/	15	/	815			F	2013-01-01
C07K	/	14	/	39			I	2013-01-01
C12N	/	15	/	81			I	2013-01-01
C07K	/	14	/	415			I	2013-01-01
C07K	/	14	/	805			I	2013-01-01
C12N	/	1	/	16			I	2013-01-01
C12N	/	15	/	635			I	2013-01-01

CPC Combination Sets									
Symbol							**Type**	**Set**	**Ranking**	**Version**
	/		/							

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	14	
/JAMES S KETTER/ Primary Examiner, Art Unit 1636	10 August 2018	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	NONE

U.S. Patent and Trademark Office — Part of Paper No.: 20180810

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/678,342	Shankar et al.
	Examiner	**Art Unit**
	JAMES S KETTER	1636

INTERNATIONAL CLASSIFICATION				
CLAIMED				
C12N	/	15	/	81

NON-CLAIMED				
	/		/	

US ORIGINAL CLASSIFICATION	
CLASS	**SUBCLASS**

CROSS REFERENCES(S)						
CLASS	**SUBCLASS (ONE SUBCLASS PER BLOCK)**					

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	14	
/JAMES S KETTER/ Primary Examiner, Art Unit 1636	10 August 2018	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	NONE

U.S. Patent and Trademark Office — Part of Paper No.: 20180810

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/678,342	Shankar et al.
	Examiner	**Art Unit**
	JAMES S KETTER	1636

☑ Claims renumbered in the same order as presented by applicant ☐ CPA ☑ T.D. ☐ R.1.47

CLAIMS

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original

NONE (Assistant Examiner)	(Date)	**Total Claims Allowed:** 14	
/JAMES S KETTER/ Primary Examiner, Art Unit 1636 (Primary Examiner)	10 August 2018 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure NONE

U.S. Patent and Trademark Office — Part of Paper No.: 20180810

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/678,342	Shankar et al.
	Examiner	**Art Unit**
	JAMES S KETTER	1636

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

CLAIMS

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☑ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	08/10/2018								
	1	=								
	2	=								
	3	=								
	4	=								
	5	=								
	6	=								
	7	=								
	8	=								
	9	=								
	10	=								
	11	=								
	12	=								
	13	=								
	14	=								

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/678,342	Shankar et al.
	Examiner	Art Unit
	JAMES S KETTER	1636

CPC - Searched*		
Symbol	**Date**	**Examiner**
No classified search made.	11/29/2017	JK
No classified search made.	06/08/2018	JK
No classified search made.	08/10/2018	JK

CPC Combination Sets - Searched*		
Symbol	**Date**	**Examiner**

US Classification - Searched*			
Class	**Subclass**	**Date**	**Examiner**

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	**Date**	**Examiner**
Inventors' names searched.	11/29/2017	JK
Priority checked.	11/29/2017	JK
BRS search attached,	11/29/2017	JK
[MEDLINE search terms and strategy as for BRS search.]	11/29/2017	JK
Updated from previous searches.	06/08/2018	JK
Updated from previous searches.	08/10/2018	JK

Interference Search			
US Class/CPC Symbol	**US Subclass/CPC Group**	**Date**	**Examiner**
	Interference search part of BRS search (US-PGPUB).	08/10/2018	JK

/JAMES S KETTER/ Primary Examiner, Art Unit 1636	

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/678,342	Shankar et al.
	Examiner	Art Unit
	JAMES S KETTER	1636

/JAMES S KETTER/ Primary Examiner, Art Unit 1636	

Sheet 1 of 1

Substitute Disclosure Form (37 CFR §1.98(b))	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No.	Application No.
Information Disclosure Statement by Applicant (Use several sheets if necessary)		38767-0048002	15/678,342
		Applicant: Impossible Foods Inc.	
		Filing Date: August 16, 2017	Group Art Unit: 1636

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1.	2018/0127764	05/10/2018	Shankar et al.			

Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
	--	--	--	--				

Other Documents (include Author, Title, Date, and Place of Publication)

Examiner Initial	Desig. ID	Document
	--	--

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.S.K/

Examiner Signature	Date Considered
/JAMES S KETTER/	08/10/2018

EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Substitute Disclosure Form

Sheet 1 of 1

Substitute Disclosure Form (37 CFR §1.98(b))	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No.	Application No.
Information Disclosure Statement by Applicant (Use several sheets if necessary)		38767-0048002	15/678,342
		Applicant: Impossible Foods Inc.	
		Filing Date: August 16, 2017	Group Art Unit: 1636

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	--	--	--	--			

Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
	--	--	--	--				

Other Documents (include Author, Title, Date, and Place of Publication)

Examiner Initial	Desig. ID	Document
	1.	~~[No Author Listed] Impossible Foods Inc. "GRAS Notification for Soybean Leghemoglobin Protein Derived from Pichia Pastoris." GRAS notice 000737, Retrieved from internet <URL:https://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm, 1063 pages~~

Examiner Signature	Date Considered
/JAMES S KETTER/	08/10/2018

EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Substitute Disclosure Form

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.S.K/

Attorney Docket No.: 38767-0048002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Smita Shankar	Art Unit	:	1636
Application No.	:	15/678,342	Examiner	:	James S. Ketter
Filed	:	August 16, 2017	Conf. No.	:	1018
Title	:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST			

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Please consider the references listed on the enclosed Form PTO-1449. Foreign patent documents and non-patent literature are enclosed; U.S. patents and patent application publications will be provided upon request.

Applicant also draws the Examiner's attention to the following application. Applicant requests the Examiner review the file history of this application, including the pending claims, which the Examiner can readily access on the USPTO's PAIR website.

Application No.	Filing Date	Inventor	Status
15/573,300	November 10, 2017	Shankar et al.	Published

This statement is being filed after a Final Office Action but before receipt of a Notice of Allowance. A fee in the amount of $240 as required under 37 CFR § 1.17(p) is being paid concurrently with this statement. Please apply any other charges, or credits, to Deposit Account No. 06-1050, referencing the above-indicated Attorney Docket Number.

Please contact the undersigned if there are any questions regarding this statement.

Respectfully submitted,

Date: /June 19, 2018/

/M. Angela Parsons/
M. Angela Parsons, Ph.D.
Reg. No. 44,282

Customer Number 26191
Fish & Richardson P.C.
Telephone: (612) 335-5070
Facsimile: (877) 769-7945
61702808.doc

08/10/2018

/JAMES S KETTER/
ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.S.K/

OK TO ENTER: /J.S.K/

08/10/2018

Attorney Docket No.: 38767-0048002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Smita Shankar	Art Unit	:	1636
Serial No.	:	15/678,342	Examiner	:	James S. Ketter
Filed	:	August 16, 2017	Conf. No.	:	1018

Title : EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST

MAIL STOP AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

RESPONSE TO OFFICE ACTION MAILED JUNE 13, 2018

In response to the Office Action mailed June 13, 2018, please consider the following reply.

Bibliographic Data

Application No: 15/678,342

Foreign Priority claimed: ○ Yes ◉ No

35 USC 119 (a-d) conditions met: ☐ Yes ☑ No ☐ Met After Allowance

Verified and Acknowledged: /JAMES S KETTER/ (Examiner's Signature) | (Initials)

Title: EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST

FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
08/16/2017	435	1636	38767-0048002
RULE			

APPLICANTS

Impossible Foods Inc., Redwood City, CA,

INVENTORS

Smita Shankar Millbrae, CA, UNITED STATES

Martin Andrew Hoyt San Francisco, CA, UNITED STATES

CONTINUING DATA

This application is a CON of PCT/US2016/031797 05/11/2016

PCT/US2016/031797 has PRO of 62313491 03/25/2016

PCT/US2016/031797 has PRO of 62236506 10/02/2015

PCT/US2016/031797 has PRO of 62222388 09/23/2015

PCT/US2016/031797 has PRO of 62220366 09/18/2015

PCT/US2016/031797 has PRO of 62203052 08/10/2015

PCT/US2016/031797 has PRO of 62185921 06/29/2015

PCT/US2016/031797 has PRO of 62183074 06/22/2015

PCT/US2016/031797 has PRO of 62159899 05/11/2015

FOREIGN APPLICATIONS

IF REQUIRED, FOREIGN LICENSE GRANTED**

08/29/2017

STATE OR COUNTRY

UNITED STATES

ADDRESS

FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MN 55440-1022

UNITED STATES

FILING FEE RECEIVED

$5,740

UNITED STATES PATENT AND TRADEMARK OFFICE
COMMISSIONER FOR PATENTS
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ALEXANDRIA VA 22313-1451

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FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MN 55440-1022
UNITED STATES OF AMERICA

Courtesy Reminder for
Application Serial No: 15/678,342

Attorney Docket No: 38767-0048002
Customer Number: 26191
Date of Electronic Notification: 08/21/2018

This is a courtesy reminder that new correspondence is available for this application. If you have not done so already, please review the correspondence. The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

An email notification regarding the correspondence was sent to the following email address(es) associated with your customer number:
PATDOCTC@fr.com

To view your correspondence online or update your email addresses, please visit us anytime at **https://sportal.uspto.gov/secure/myportal/privatepair.** If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov or call 1-866-217-9197.

Attorney Docket No.: 38767-0048002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Smita Shankar	Art Unit	:	1636
Application No.	:	15/678,342	Examiner	:	James S. Ketter
Filed	:	August 16, 2017	Conf. No.	:	1018
Title	:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST			

MAIL STOP ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Please consider the documents listed on the disclosure form submitted with this statement. Copies of the documents are being submitted with this statement to the extent required under 37 C.F.R. § 1.98(a)(2). A copy of a Communication from a foreign Patent Office in a counterpart application also is being submitted.

This statement is being filed after a Final Office Action or a Notice of Allowance, but before payment of the Issue Fee.

Under 37 C.F.R. § 1.97(e)(1), Applicant certifies that each item of information contained in this Information Disclosure Statement was first cited in a Communication from a foreign Patent Office in a counterpart foreign application not more than three months prior to the filing of this Information Disclosure Statement.

Please apply any charges under 37 C.F.R. § 1.17(p), and any credits, to Deposit Account No. 06-1050, referencing the above-indicated Attorney Docket Number.

Please contact the undersigned if there are any questions regarding this statement.

Respectfully submitted,

Date: /October 9, 2018/

/M. Angela Parsons/
M. Angela Parsons, Ph.D.
Reg. No. 44,282

Customer Number 26191
Fish & Richardson P.C.
Telephone: (612) 335-5070
Facsimile: (877) 769-7945
61765331.doc

Sheet 1 of 1

Substitute Disclosure Form	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No.	Application No.
Information Disclosure Statement by Applicant (Use several sheets if necessary)		38767-0048002	15/678,342
		Applicant: Impossible Foods Inc.	
(37 CFR §1.98(b))		Filing Date: August 16, 2017	Group Art Unit: 1636

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate

Foreign Patent Documents or Published Foreign Patent Applications								
Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
	1.	WO 2015/153666	10/8/2015	WIPO				

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
	2.	Krainer et al., "Optimizing cofactor availability for the production of recombinant heme peroxidase in Pichia pastoris", MICROBIAL CELL FACTORIES, vol. 14, no. 1, 13 January 2015
	3.	Liu et al., "Balanced globin protein expression and heme biosynthesis improve production of human hemoglobin in Saccharomyces cerevisiae", METABOLIC ENGINEERING, vol. 21, pages 9-16, 2014
	4.	Fraser et al., "Safety Evaluation of Soy Leghemoglobin Protein Preparation Derived From Pichia pastoris , Intended for Use as a Flavor Catalyst in Plant-Based Meat", INTERNATIONAL JOURNAL OF TOXICOLOGY, vol. 37, no. 3, 11 April 2018, pages 241-262

Examiner Signature	Date Considered

EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Substitute Disclosure Form

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(54) Title: GROUND MEAT REPLICAS

(57) Abstract: This document relates to ground meat replicas, and more particularly to plant-based products that mimic ground meat, including the fibrousness, heterogeneity in texture, beefy flavor, and red-to-brown color transition during cooking of ground meat. For example, this document provides meat replicas that include proteins that are selected based upon the temperature at which they gel and/or denature to replicate the behavior and qualities of meat during cooking, i.e., the firming, syneresis (water release), chew texture, or mouthfeel.

GROUND MEAT REPLICAS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Application Serial No. 61/973,181, filed on March 31, 2014, and to U.S. Application Serial No. 62/058,230, filed on October 1, 2014, the disclosure of which are incorporated by reference in their entirety.

TECHNICAL FIELD

This disclosure relates to meat replicas, such as ground meat replicas, and more particularly to plant-based products that mimic the texture, appearance, and sensory aspects of ground meat, including the texture, appearance, and sensory aspects of cooking and eating ground meat, such as the fibrousness, heterogeneity in texture, beefy flavor, and red-to-brown color transition during cooking of ground meat. This disclosure also relates to compositions and methods for altering the flavor of a food product or a food replica, such as a cheese or meat replica.

BACKGROUND

Meat substitute compositions typically are extruded soy/grain mixtures that fail to replicate the experience of cooking and eating meat. Common limitations of plant-based meat substitute products include a texture and mouth-feel more homogenous than that of equivalent meat products. Furthermore, as these products must largely be sold pre-cooked, with artificial flavors and aromas pre-incorporated, they fail to replicate the aromas, flavors, and other key features, such as texture and mouth-feel, associated with cooking or cooked meat, and they also may have added off flavors. As a result, these products mainly appeal to a limited consumer base that is already committed to vegetarianism, but have failed to appeal to the larger consumer segment accustomed to eating meat. It would be useful to have improved plant-based meat substitutes, which better replicate the fibrousness, texture, aromas and flavors of meat during and/or after cooking.

SUMMARY

This document is based on methods and materials for making plant-based products that can mimic ground meat, including the fibrousness, heterogeneity in texture, beefy or other meat flavor, and red-to-brown color transition during cooking of ground meat, without off flavors. For example, this document provides meat replicas that include proteins that are selected based upon the temperature at which they gel and/or denature to replicate the behavior and qualities of meat during cooking, i.e., the firming, syneresis (water release), chew texture, or mouthfeel. For example, the temperature of denaturing and gelling of the proteins selected to be in the meat replica can be similar to that of proteins typically found in meat (e.g., actin and myosin). Further the plant-based products provided herein can include flavoring agents (e.g., flavorings, flavoring precursors, and/or flavoring compounds) that can provide meaty flavors, such that a plant-based meat replica has a more natural flavor and does not have off flavors. This document therefore also provides methods for making plant-based products containing such flavoring agents.

In one aspect, this document features a meat replica composition that includes about 5% to about 88% (e.g., about 40% to about 88%, about 45% to about 60%, or about 15% to about 55%) by weight of a meat dough; about 0% to about 40% (e.g., about 1% to about 30%, about 5% to about 25%, or about 15% to about 25%) by weight of a carbohydrate-based gel; about 5% to about 35% (e.g., about 10% to about 15%, about 12% to about 18%, or about 20% to about 25%) by weight of a fat; about 0.00001% to about 10% (e.g., about 3% to about 7%, about 0.001% to about 2%, or about 0.00001% to about 2%) by weight of a flavoring agent; about 0% to about 15% (e.g., about 2% to about 15% or about 2% to about 10%) by weight of a binding agent; and about 0.01% to about 4% (e.g., about 0.05% to about 1%, or about 0.5% to about 2%) by weight of a heme-containing protein and/or an iron salt. The meat dough can include a flavoring agent. The fat can include a flavoring agent. The meat dough can be about 45% to about 60% by weight of the composition. The carbohydrate-based gel can be about 10% to about 25% by weight of the composition. The fat can be about 10% to about 15% by weight of the composition. The flavoring agent can be about 3% to about 7% or about

0.001% to about 2% by weight of the composition. The flavoring agent can include one or more flavor precursors, a flavoring, or a flavoring compound. The flavoring agent can be a combination of a flavoring and one or more flavor precursors. The binding agent can be about 2% to about 10% by weight of the composition. The binding agent can include one or more proteins that have been chemically or enzymatically modified to improve their textural and/or flavor properties, or to modify their denaturation and gelling temperatures. The heme-containing protein can be about 0.01% to about 2% by weight of the composition. The composition can include the heme-containing protein and the iron salt. The meat dough can include an isolated plant protein, an edible fibrous component, an optional flavoring agent, and an optional fat. The binding agent can be a conglycinin protein.

In another aspect, this document features a meat replica composition that includes about 5% to about 80% (e.g., about 20% to about 30%) by weight of a meat dough; about 5% to about 35% (e.g., about 15% to about 25%) by weight of a fat; about 15% to about 40% (e.g., about 15% to about 25%) by weight of an edible fibrous component; about 0.1% to about 18% (e.g., about 7% to about 18%) by weight of a carbohydrate-based gel; about 0% to about 10% (e.g., about 0% to about 10%) by weight of a flavoring agent; about 0.5% to about 15% (e.g., about 5% to about 15%) by weight of a binding agent; and about 0.1% to about 8% (e.g., about 2% to about 8%) by weight of a heme-containing protein and/or an iron salt.

In another aspect, this document features a method of making a ground meat replica. The method can include (a) heating a dough to a temperature ranging from 150°F to 250°F, the dough comprising an isolated plant protein, an optional edible fibrous component, one or more optional flavoring agents, and an optional fat; (b) combining the dough, after heating, with a fat, the fat optionally containing a flavoring agent and/or an isolated plant protein; and (c) combining the dough from step (b) with a carbohydrate-based gel, an optional edible fibrous component, an optional binding agent, a highly conjugated heterocyclic ring complexed to an iron ion and/or an iron salt, and one or more optional flavoring agents to make the ground meat replica. The method can further include breaking the dough from step (b) into pieces before combining with the

carbohydrate-based gel, the optional edible fibrous component, the optional binding agent, the highly conjugated heterocyclic ring complexed to an iron ion and/or the iron salt, and one or more optional flavoring agents.

In another aspect, this document features a method of flavoring a meat dough. The method can include (a) combining a first highly conjugated heterocyclic ring complexed to an iron ion and/or a first iron salt with one or more flavor precursors and an optional fat; (b) heating the mixture to form one or more flavor compounds; and (c) making a dough comprising an isolated plant protein, an optional edible fibrous component, and the mixture from step (b). The method can further include (d) combining the dough, after heating, with a fat, the fat optionally containing a flavoring agent and/or an isolated plant protein; and (e) combining the dough of step (d) with a carbohydrate-based gel, an optional binding agent, a second highly conjugated heterocyclic ring complexed to an iron ion and/or a second iron salt, and one or more optional flavoring agents to make a ground meat replica. The method can further include breaking the dough from step (d) into pieces before combining with the carbohydrate-based gel, the optional binding agent, the second highly conjugated heterocyclic ring complexed to an iron ion and/or the second iron salt, and one or more optional flavoring agents.

In another aspect, this document features a method of flavoring a meat dough, where the method includes (a) making a dough comprising an isolated plant protein, an optional edible fibrous component, one or more optional flavoring agents, and an optional fat; (b) making a flavored fat by combining a fat with a highly conjugated heterocyclic ring complexed to an iron ion and/or a first iron salt, and one or more flavor precursors and heating the mixture; and (c) combining the dough, after heating, with the flavored fat. The method can further include combining the dough of step (c) with a carbohydrate-based gel, an optional binding agent, a second highly conjugated heterocyclic ring complexed to an iron ion and/or a second iron salt, and one or more optional flavoring agents to make a ground meat replica. The method can further include breaking the dough of step (c) before combining with the carbohydrate-based gel, the optional binding agent, the second highly conjugated heterocyclic ring complexed to an iron ion and/or the second iron salt, and one or more optional flavoring agents.

This document also features a method of making a ground meat replica, where the method includes (a) combining an iron salt with one or more flavor precursors and an optional fat; (b) heating the mixture to form one or more flavor compounds; (c) making a dough comprising an isolated plant protein, an optional edible fibrous component, and the mixture from step (b); (d) combining the dough, after heating, with a fat, the fat optionally containing a flavoring agent and/or an isolated plant protein; and (e) combining the dough of step (d) with a carbohydrate-based gel, an optional binding agent, an iron salt, an optional highly conjugated heterocyclic ring complexed to an iron ion, and one or more optional flavoring agents to make the ground meat replica. The method can further include breaking the dough from step (d) into pieces before combining with the carbohydrate-based gel, the optional binding agent, the iron salt, the optional highly conjugated heterocyclic ring complexed to an iron ion, and one or more optional flavoring agents. In some embodiments, a highly conjugated heterocyclic ring complexed to an iron ion can be combined with the iron salt, the one or more flavor precursors, and the fat before heating the mixture.

In yet another aspect, this document features a method of making a ground meat replica. The method can include (a) making a dough comprising an isolated plant protein, an optional edible fibrous component, one or more optional flavoring agents, and an optional fat; (b) making a flavored fat by combining a fat with an iron salt and one or more flavor precursors and heating the mixture; (c) combining the dough, after heating, with the flavored fat; and (d) combining the dough of step (c) with a carbohydrate-based gel, an optional binding agent, an iron salt, an optional highly conjugated heterocyclic ring complexed to an iron ion, and one or more optional flavoring agents to make the ground meat replica. The method can further include breaking the dough from step (c) before combining with the carbohydrate-based gel, the optional binding agent, the iron salt, the optional highly conjugated heterocyclic ring complexed to an iron ion, and one or more optional flavoring agents. In some embodiments, a highly conjugated heterocyclic ring complexed to an iron ion can be combined with the fat, the iron salt, and the one or more flavor precursors before heating the mixture.

In any of the methods or compositions described herein, the iron salt can be iron gluconate, iron chloride, iron oxalate, iron nitrate, iron citrate, iron ascorbate, ferrous sulfate, ferric pyrophosphate, or any other aqueous soluble salt.

In any of the methods or compositions described herein, the heme-containing protein can be a non-animal heme-containing protein, such as a plant-derived heme-containing protein (e.g., leghemoglobin). Further, in some embodiments, the heme-containing protein can be isolated or isolated and purified.

In any of the methods or compositions described herein, wherein the one or more flavor precursors can be a sugar, a sugar alcohol, a sugar acid, a sugar derivative, an oil, a free fatty acid, an amino acid or derivative thereof, a nucleoside, a nucleotide, a vitamin, an acid, a peptide, a phospholipid, a protein hydrolysate, a yeast extract, or a mixture thereof. For example, the flavor precursor can be selected from the group consisting of glucose, fructose, ribose, arabinose, glucose-6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, inositol, maltose, sucrose, maltodextrin, glycogen, nucleotide-bound sugars, molasses, a phospholipid, a lecithin, inosine, inosine monophosphate (IMP), guanosine monophosphate (GMP), pyrazine, adenosine monophosphate (AMP), lactic acid, succinic acid, glycolic acid, thiamine, creatine, pyrophosphate, vegetable oil, algal oil, sunflower oil, corn oil, soybean oil, palm fruit oil, palm kernel oil, safflower oil, flaxseed oil, rice bran oil, cottonseed oil, olive oil, sunflower oil, canola oil, flaxseed oil, coconut oil, mango oil, a free fatty acid, cysteine, methionine, isoleucine, leucine, lysine, phenylalanine, threonine, tryptophan, valine, arginine, histidine, alanine, asparagine, aspartate, glutamate, glutamine, glycine, proline, serine, tyrosine, glutathione, an amino acid derivative, urea, pantothenic acid, ornithine, niacin, glycerol, citrulline, taurine, biotin, borage oil, fungal oil, blackcurrant oil, betaine, beta carotene, B-vitamins, N-Acetyl L-cysteine, iron glutamate and a peptone, or mixtures thereof.

In any of the methods or compositions described herein, the isolated plant protein in the dough can include wheat gluten, a dehydrin protein, an albumin, a globulin, or a zein, or mixtures thereof.

In any of the methods or compositions described herein, the optional edible fibrous component can include plant fibers from carrot, bamboo, pea, broccoli, potato,

sweet potato, corn, whole grains, alfalfa, kale, celery, celery root, parsley, cabbage, zucchini, green beans, kidney beans, black beans, red beans, white beans, beets, cauliflower, nuts, apple skins, oats, wheat, or psyllium, or a mixture thereof.

In any of the methods or compositions described herein, the edible fibrous component can include an extruded mixture of isolated plant proteins. The extruded mixture can contain wheat gluten and soy protein isolate, and optionally can further contain a flavoring agent (e.g., a flavoring such as yeast extract, a protein hydrolysate, or an oil; a flavor compound; or a flavor precursor). In some embodiments, the edible fibrous component can be a solution-spun protein fiber (e.g., a solution-spun protein fiber containing a prolamin such as corn zein, pea prolamin, kafirin, secalin, hordein, avenin, or a mixture thereof).

In any of the methods or compositions described herein, the fat can be a non-animal fat, an animal fat, or a mixture of non-animal and animal fat. The fat can be an algal oil, a fungal oil, corn oil, olive oil, soy oil, peanut oil, walnut oil, almond oil, sesame oil, cottonseed oil, rapeseed oil, canola oil, safflower oil, sunflower oil, flax seed oil, palm oil, palm kernel oil, coconut oil, babassu oil, shea butter, mango butter, cocoa butter, wheat germ oil, borage oil, black currant oil, sea-buckhorn oil, macadamia oil, saw palmetto oil, conjugated linoleic oil, arachidonic acid enriched oil, docosahexaenoic acid (DHA) enriched oil, eicosapentaenoic acid (EPA) enriched oil, palm stearic acid, sea-buckhorn berry oil, macadamia oil, saw palmetto oil, or rice bran oil; or margarine or other hydrogenated fats. In some embodiments, for example, the fat is algal oil. The fat can contain the flavoring agent and/or the isolated plant protein (e.g., a conglycinin protein).

In any of the methods or compositions described herein, the dough can include the flavoring agent. In any of the methods or compositions, the non-animal fat in the dough can include a flavoring agent. The flavoring agent can be selected from the group consisting of a vegetable extract, a fruit extract, an acid, an antioxidant, a carotenoid, a lactone, and combinations thereof. The antioxidant can be epigallocatechin gallate. The carotenoid can be lutein, β-carotene, zeaxanthin, trans-β-apo-8'-carotenal, lycopene, or

canthaxanthin. The vegetable extract can be from a cucumber or tomato. The fruit extract can be from a melon or pineapple.

In any of the methods or compositions described herein, the carbohydrate based gel can have a melting temperature between about 45°C and about 85°C. The carbohydrate-based gel can include agar, pectin, carrageenan, konjac, alginate, chemically-modified agarose, or mixtures thereof.

In any of the methods or compositions described herein, the ground meat replica can contain the binding agent. The binding agent can be an isolated plant protein (e.g., a RuBisCO, an albumin, a gluten, a conglycinin, or mixtures thereof). The denaturation temperature of the binding agent can be between about 40°C and about 80°C. The binding agent can be a carbohydrate based gel that becomes firm upon cooking to 140°F to 190°F. The carbohydrate based gel can contain methylcellulose, hydroxypropylmethyl cellulose, guar gum, locust bean gum, xanthan gum, or a mixture thereof. The binding agent can be egg albumin or collagen.

In any of the methods or compositions described herein, the highly conjugated heterocyclic ring complexed to an iron ion can be a heme moiety, or a porphyrin, porphyrinogen, corrin, corrinoid, chlorin, bacteriochlorophyll, corphin, chlorophyllin, bacteriochlorin, or isobacteriochlorin moiety complexed to an iron ion. The heme moiety can be a heme-containing protein (e.g., a non-symbiotic hemoglobin, a Hell's gate globin I, a flavohemoprotein, a leghemoglobin, a heme-dependent peroxidase, a cytochrome c peroxidase, or a mammalian myoglobin). In some embodiments, the heme-containing protein can be a leghemoglobin. The leghemoglobin can be from soybean, pea, or cowpea.

In another aspect, this document features a method of increasing the meat flavor or masking off flavors from plant material in a food product. The method can include adding, to the food product, one or more lactones at a concentration of 10^{-3} to 10^{-11} of the food product, wherein the lactones are selected from the group consisting of tetrahydro-6-methyl-2H-pyran-2-one, delta-octalactone, 5-ethyldihydro-2(3H)-furanone, butyrolactone, dihydro-5-pentyl-2(3H)-furanone, dihydro-3-methylene-2,5-furandione, l-pentoyl lactone, tetrahydro-2H-pyran-2-one, 6-heptyltetrahydro-2H-pyran-2-one, γ-

octalactone, 5-hydroxymethyldihydrofuran-2-one, 5-ethyl-2(5H)-furanone, 5-acetyldihydro-2(3H)-furanone, trans-3-methyl-4-octanolide 2(5H)-furanone, 3-(1,1-dimethylethyl)-2,5-urandione, 3,4-dihydroxy-5-methyl-dihydrofuran-2-one, 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, δ-tetradecalactone, and dihydro-4-hydroxy-2(3H)-furanone. In some embodiments, the lactones can be 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, butyrolactone, γ-octalactone, and δ-tetradecalactone. The food product can be a meat replica. The meat replica can be free of animal products.

This document also features a method of increasing the meat flavor or masking off flavors from plant material in a food product, where the method includes adding, to the food product, one or more carotenoids at a concentration of between 0.00001% and 0.1% of the food product, wherein the carotenoids are selected from the group consisting of β-carotene, zeaxanthin, lutein, trans-β-apo-8'-carotenal, lycopene, canthaxanthin, and combinations thereof. The food product can be a meat replica. The meat replica can be free of animal products.

In another embodiment, this document features a method of increasing the meat flavor of a meat replica. The method can include adding, to the meat replica, a vegetable juice, a vegetable puree, a vegetable extract, a fruit juice, a fruit puree, or a fruit extract to the meat replica at a concentration from 0.0001% to 10% of the meat replica. The vegetable juice, vegetable puree, vegetable extract, a fruit juice, a fruit puree, or a fruit extract can be a *Cucumis* juice, puree, or extract (e.g., a juice, puree, or extract from a cucumber or a melon). The method vegetable juice, vegetable puree, vegetable extract, fruit juice, fruit puree, or fruit extract can be cooked or otherwise treated to denature proteins before adding to the meat replica. The meat replica can be free of animal products.

In another aspect, this document features a food product or food replica product containing a heme-containing protein and one or more lactones at a concentration of 10^{-3} to 10^{-11} of the food product, wherein the one or more lactones are selected from the group consisting of tetrahydro-6-methyl-2H-pyran-2-one, delta-octalactone, 5-ethyldihydro-2(3H)-furanone, butyrolactone, dihydro-5-pentyl-2(3H)-furanone, dihydro-3-methylene-2,5-furandione, l-pentoyl lactone, tetrahydro-2H-pyran-2-one, 6-heptyltetrahydro-2H-

pyran-2-one, γ-octalactone, 5-hydroxymethyldihydrofuran-2-one, 5-ethyl-2(5H)-furanone, 5-acetyldihydro-2(3H)-furanone, trans-3-methyl-4-octanolide 2(5H)-furanone, 3-(1,1-dimethylethyl)-2,5-urandione, 3,4-dihydroxy-5-methyl-dihydrofuran-2-one, 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, δ-tetradecalactone, and dihydro-4-hydroxy-2(3H)-furanone. For example, the one or more lactones can be 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, butyrolactone, γ-octalactone, and δ-tetradecalactone. The food product or food replica product can be a meat replica. The meat replica can be free of animal products.

This document also features a food product or food replica product containing a heme-containing protein and one or more carotenoids at a concentration of between 0.00001% and 0.1% of the food product, wherein the one or more carotenoids are selected from the group consisting of β-carotene, zeaxanthin, lutein, trans-β-apo-8'-carotenal, lycopene, canthaxanthin, and combinations thereof. The food product or food replica product can be a meat replica. The meat replica can be free of animal products.

In another aspect, this document features a food product or food replica product containing (a) a heme-containing protein, and (b) a vegetable juice, a vegetable puree, a vegetable extract, a fruit juice, a fruit puree, or a fruit extract at a concentration from 0.0001% to 10% of the food product. The vegetable juice, vegetable puree, vegetable extract, a fruit juice, a fruit puree, or a fruit extract can be a *Cucumis* juice, puree, or extract. The *Cucumis* juice, puree, or extract can be from a cucumber or a melon. The vegetable juice, vegetable puree, vegetable extract, fruit juice, fruit puree, or fruit extract can have been cooked or otherwise treated to denature proteins before being added to the food replica product. For example, the vegetable juice, vegetable puree, vegetable extract, fruit juice, fruit puree, or fruit extract can have been heated to a temperature of about 60°C to about 100°C before being added to the food replica product. The food product can be free of animal products.

In another aspect, this document features a food replica product containing one or more lactones at a concentration of 10^{-3} to 10^{-11} of the food product, wherein the one or more lactones are selected from the group consisting of tetrahydro-6-methyl-2H-pyran-2-one, delta-octalactone, 5-ethyldihydro-2(3H)-furanone, butyrolactone, dihydro-5-pentyl-

2(3H)-furanone, dihydro-3-methylene-2,5-furandione, l-pentoyl lactone, tetrahydro-2H-pyran-2-one, 6-heptyltetrahydro-2H-pyran-2-one, γ-octalactone, 5-hydroxymethyldihydrofuran-2-one, 5-ethyl-2(5H)-furanone, 5-acetyldihydro-2(3H)-furanone, trans-3-methyl-4-octanolide 2(5H)-furanone, 3-(1,1-dimethylethyl)-2,5-urandione, 3,4-dihydroxy-5-methyl-dihydrofuran-2-one, 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, δ-tetradecalactone, and dihydro-4-hydroxy-2(3H)-furanone. The one or more lactones can be 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, butyrolactone, γ-octalactone, and δ-tetradecalactone.

In still another aspect, this document features a food replica product containing one or more carotenoids at a concentration of between 0.00001% and 0.1% of the food product, wherein the one or more carotenoids are selected from the group consisting of β-carotene, zeaxanthin, lutein, trans-β-apo-8'-carotenal, lycopene, canthaxanthin, and combinations thereof.

This document also features a food replica product containing a vegetable juice, a vegetable puree, a vegetable extract, a fruit juice, a fruit puree, or a fruit extract at a concentration from 0.0001% to 10% of the food product. The vegetable juice, vegetable puree, vegetable extract, fruit juice, fruit puree, or fruit extract can be a *Cucumis* juice, puree, or extract (e.g., a *Cucumis* juice, puree, or extract from a cucumber or a melon). The vegetable juice, vegetable puree, vegetable extract, fruit juice, fruit puree, or fruit extract can have been cooked or otherwise treated to denature proteins before being added to the food replica product. For example, the vegetable juice, vegetable puree, vegetable extract, fruit juice, fruit puree, or fruit extract can have been heated to a temperature of about 60°C to about 100°C before being added to the food replica product.

In some embodiments, the food replica products provided herein can be free of animal products, wheat gluten, soy protein, and/or tofu.

Any of the food replica products provided herein can contain one or more of a meat dough, a carbohydrate-based gel, a non-animal fat, and a binding agent.

Any of the food replica products provided herein can be a meat replica. Further materials and methods for making meat replicas can be found in, for example, U.S.

Publication No. 2014/0193547, and PCT publications WO 2014/110532 and WO 2014/110539, each of which is incorporated herein by reference in its entirety.

Any of the food replica products provided herein can be a cheese replica. The cheese replica can contain a nut milk, a cross-linking enzyme, or a cheese culture. Further materials and methods for making cheese replicas can be found in, for example, U.S. Publication No. 2014/0127358, and PCT publication WO 2014/110540, both of which are incorporated herein by reference in their entirety.

In yet another aspect, this document features a ground meat replica containing (a) a dough that contains an isolated plant protein, an optional edible fibrous component, one or more optional flavoring agents, and an optional fat; (b) a fat, the fat optionally containing a flavoring agent and/or an isolated plant protein; and (c) a carbohydrate-based gel, a binding agent, a highly conjugated heterocyclic ring complexed to an iron ion and/or an iron salt, an optional edible fibrous component, and one or more optional flavoring agents. The binding agent can be an isolated plant protein (e.g., a RuBisCO, an albumin, a gluten, a conglycinin, or mixtures thereof). The denaturation temperature of the binding agent can be between about 40°C and about 80°C.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims. The word "comprising" in the claims may be replaced by "consisting essentially of" or with "consisting of," according to standard practice in patent law.

DETAILED DESCRIPTION

In general, this document provides methods and materials for producing meat replicas, including ground meat replicas (e.g., ground beef, ground chicken, ground turkey, ground lamb, or ground pork), as well as replicas of cuts of meat and fish. Broadly, the document provides methods for making ground meat replicas that include preparing a meat replica dough (referred to herein as "meat dough") that includes an optional edible fibrous component, combining the meat dough with a fat (typically a non-animal-based fat, although it is to be noted that an animal-based fat could be used) that can optionally include a flavoring agent and/or an isolated plant protein, adding a carbohydrate-based gel, an optional edible fibrous component, a binding agent, a highly conjugated heterocyclic ring complexed to an iron ion and/or an iron salt, and one or more flavoring agents to make the replica. After combining the meat dough with the fat, the mixture can be broken into smaller pieces before adding further ingredients.

The meat dough can incorporate an edible fibrous component to help achieve a textural heterogeneity and fibrousness in the meat replica that resembles the heterogeneity and texture of ground meat (e.g., ground beef). Incorporating flavoring agents into multiple components of the meat replica (e.g., two or more of the meat dough, the edible fibrous component, the non-animal-based fat, or the assembled replica), helps mimic the sensory properties of ground meat. In some embodiments, flavoring agents are incorporated into three components of the meat replica. In some embodiments, flavoring agents are incorporated into four components of the meat replica.

As described herein, the flavoring agents can be flavor precursors, flavor compounds produced from reacting flavor precursors with iron, or flavorings such as extracts (e.g., a malt extract, a yeast extract, a vegetable or fruit extract, such as a cucumber extract or a melon extract, or a peptone) or protein hydrolysates such as vegetable protein hydrolysates, soy protein hydrolysates, yeast protein hydrolysates, algal protein hydrolysates, or meat protein hydrolysates or flavor compounds, natural or synthetic. Flavor precursors can react, e.g., with the iron in a highly conjugated heterocyclic ring complexed to an iron ion or an iron salt, with each other, or with flavorings, upon heating. Accordingly, in the meat replicas described herein,

combinations of pre-cooked, *i.e.*, reacted, flavor components, uncooked flavor precursors that can react (e.g., with the iron salt and/or highly conjugated heterocyclic ring complexed to an iron ion or with each other) during cooking of the replicas, or flavorings or flavor compounds that introduce a flavor without requiring a reaction, can be incorporated into the meat replica to reproduce the sensory experience of cooking and eating cooked ground meat. The flavor and/or aroma profile of the ground meat product can be modulated by the type and concentration of the flavor precursors, the pH of the reaction, the length of cooking, the type and amount of iron complex (e.g., a heme-cofactor such as a heme-containing protein, or heme bound to non-peptidic polymer or macromolecule), the temperature of the reaction, and the amount of water activity in the product, among other factors.

A highly conjugated heterocyclic ring complexed to an iron ion is referred to herein as an iron complex. Such iron complexes include heme moieties or other highly conjugated heterocylic rings complexed to an iron ion. "Heme" refers to a prosthetic group bound to iron (Fe^{2+} or Fe^{3+}) in the center of a porphyrin ring. Thus, an iron complex can be a heme moiety, or a porphyrin, porphyrinogen, corrin, corrinoid, chlorin, bacteriochorophyll, corphin, chlorophyllin, bacteriochlorin, or isobacteriochlorin moiety complexed to an iron ion. The heme moiety can be a heme cofactor such as a heme-containing protein; a heme moiety bound to a non-peptidic polymer or other macromolecule such as a liposome, a polyethylene glycol, a carbohydrate, a polysaccharide, or a cyclodextrin.

In some embodiments, the iron complex is a heme-containing protein that is isolated and purified. As used herein, the term "isolated and purified" with respect to a protein or a protein fraction indicates that the protein or protein fraction has been separated from other components of the source material (e.g., other animal, plant, fungal, algal, or bacterial proteins), such that the protein or protein fraction is at least 50% (e.g., at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99%) free, by dry weight, of the other components of the source material.

As used herein, an "enriched" protein or protein fraction composition is at least 2-fold (e.g., at least 3-fold, 4-fold, 5-fold, 10-fold, 20-fold, 50-fold, or 100-fold) enriched in that protein or protein fraction relative to the source material.

The term "heme containing protein" can be used interchangeably with "heme containing polypeptide" or "heme protein" or "heme polypeptide" and includes any polypeptide that can covalently or noncovalently bind a heme moiety. In some embodiments, the heme-containing polypeptide is a globin and can include a globin fold, which comprises a series of seven to nine alpha helices. Globin type proteins can be of any class (e.g., class I, class II, or class III), and in some embodiments, can transport or store oxygen. For example, a heme-containing protein can be a non-symbiotic type of hemoglobin or a leghemoglobin. A heme-containing polypeptide can be a monomer, i.e., a single polypeptide chain, or can be a dimer, a trimer, tetramer, and/or higher order oligomer. The life-time of the oxygenated Fe^{2+} state of a heme-containing protein can be similar to that of myoglobin or can exceed it by 10%, 20%, 30%, 50%, 100% or more under conditions in which the heme-protein-containing consumable is manufactured, stored, handled or prepared for consumption. The life-time of the unoxygenated Fe^{2+} state of a heme-containing protein can be similar to that of myoglobin or can exceed it by 10%, 20%, 30%, 50%, 100% or more under conditions in which the heme-protein-containing consumable is manufactured, stored, handled or prepared for consumption

Non-limiting examples of heme-containing polypeptides can include an androglobin, a cytoglobin, a globin E, a globin X, a globin Y, a hemoglobin, a myoglobin, an erythrocruorin, a beta hemoglobin, an alpha hemoglobin, a protoglobin, a cyanoglobin, a cytoglobin, a histoglobin, a neuroglobins, a chlorocruorin, a truncated hemoglobin (e.g., HbN or HbO), a truncated 2/2 globin, a hemoglobin 3 (e.g., Glb3), a cytochrome, or a peroxidase.

Heme-containing proteins that can be used in the ground meat replicas described herein can be from mammals (e.g., farms animals such as cows, goats, sheep, pigs, ox, or rabbits), birds, plants, algae, fungi (e.g., yeast or filamentous fungi), ciliates, or bacteria. For example, a heme-containing protein can be from a mammal such as a farm animal (e.g., a cow, goat, sheep, pig, fish, ox, or rabbit) or a bird such as a turkey or chicken.

Heme-containing proteins can be from a plant such as *Nicotiana tabacum* or *Nicotiana sylvestris* (tobacco); *Zea mays* (corn), *Arabidopsis thaliana*, a legume such as *Glycine max* (soybean), *Cicer arietinum* (garbanzo or chick pea), *Pisum sativum* (pea) varieties such as garden peas or sugar snap peas, *Phaseolus vulgaris* varieties of common beans such as green beans, black beans, navy beans, northern beans, or pinto beans, *Vigna unguiculata* varieties (cow peas), *Vigna radiata* (mung beans), *Lupinus albus* (lupin), or *Medicago sativa* (alfalfa); *Brassica napus* (canola); *Triticum sps.* (wheat, including wheat berries, and spelt); *Gossypium hirsutum* (cotton); *Oryza sativa* (rice); *Zizania sps.* (wild rice); *Helianthus annuus* (sunflower); *Beta vulgaris* (sugarbeet); *Pennisetum glaucum* (pearl millet); *Chenopodium sp.* (quinoa); *Sesamum sp.* (sesame); *Linum usitatissimum* (flax); or *Hordeum vulgare* (barley). Heme-containing proteins can be isolated from fungi such as *Saccharomyces cerevisiae*, *Pichia pastoris*, *Magnaporthe oryzae*, *Fusarium graminearum*, *Aspergillus oryzae*, *Trichoderma reesei*, *Myceliopthera thermophile*, *Kluyveramyces lactis*, or *Fusarium oxysporum*. Heme-containing proteins can be isolated from bacteria such as *Escherichia coli*, *Bacillus subtilis, Bacillus licheniformis, Bacillus megaterium, Synechocistis sp., Aquifex aeolicus, Methylacidiphilum infernorum*, or thermophilic bacteria such as *Thermophilus spp*. The sequences and structure of numerous heme-containing proteins are known. *See for example*, Reedy, *et al.*, *Nucleic Acids Research*, 2008, Vol. 36, Database issue D307–D313 and the Heme Protein Database available on the world wide web at http://hemeprotein.info/heme.php.

In some embodiments, a non-symbiotic hemoglobin can be from any plant. In some embodiments, a non-symbiotic hemoglobin can be from a plant selected from the group consisting of soybean, sprouted soybean, alfalfa, golden flax, black bean, black eyed pea, northern bean, tobacco, pea, garbanzo, moong bean, cowpeas, pinto beans, pod peas, quinoa, sesame, sunflower, wheat berries, spelt, barley, wild rice, and rice.

In some embodiments, a leghemoglobin can be a soy, pea, or cowpea leghemoglobin.

In some embodiments, isolated plant proteins are used. As used herein, the term "isolated" with respect to a protein or a protein fraction (e.g., a 7S fraction) indicates that the protein or protein fraction has been separated from other components of the source

material (e.g., other animal, plant, fungal, algal, or bacterial proteins), such that the protein or protein fraction is at least 2% (e.g., at least 5%, 10%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99%) free, by dry weight, of the other components of the source material. Thus, in some embodiments, the iron complex can be a heme-containing protein (e.g., a plant heme-containing protein) that is isolated. Proteins can be separated on the basis of their molecular weight, for example, by size exclusion chromatography, ultrafiltration through membranes, or density centrifugation. In some embodiments, the proteins can be separated based on their surface charge, for example, by isoelectric precipitation, anion exchange chromatography, or cation exchange chromatography. Proteins also can be separated on the basis of their solubility, for example, by ammonium sulfate precipitation, isoelectric precipitation, surfactants, detergents or solvent extraction. Proteins also can be separated by their affinity to another molecule, using, for example, hydrophobic interaction chromatography, reactive dyes, or hydroxyapatite. Affinity chromatography also can include using antibodies having specific binding affinity for the heme-containing protein, nickel nitroloacetic acid (NTA) for His-tagged recombinant proteins, lectins to bind to sugar moieties on a glycoprotein, or other molecules which specifically binds the protein.

Example 2 describes a method for isolating RuBisCO from a plant (e.g., spinach or alfalfa). The extraction process can be improved further by adding reductants such as metabisulfite (about 2% w/v solution or more) to the initial extraction buffer and maintaining anaerobic conditions through the process and/or by adding 0.05-1% v/v cationic flocculants such as Superfloc 781G,Magnafloc LT 7989 (BASF), or Tramfloc 863A to the extraction buffer to the extraction buffer. The resuspended protein pellet from such methods, upon microfiltration at a pH of 7.0, would still perform, provide the same color, and have the same denaturation properties.

Example 4 describes a method for isolating conglycinin (also can be referred to as a 7S fraction) from a plant such as soybean. Other sources of 7S include seeds such as, without limitation, peas, chickpeas, mung beans, kidney beans, fava beans, cowpeas, pine nuts, rice, corn, and sesame. Soluble proteins can be extracted from defatted soybean

flour, and then the mixture acidified (e.g., to a pH of 4.5) to precipitate the proteins. Conglycinin can be resolubilized and concentrated, e.g., using ultrafiltration.

In some embodiments, the isolated protein is decolorized. For example, the RuBisCO concentrates can be decolorized (pH 7-9) by passing over columns packed with activated carbon. The colorants can bind to the column while RuBisCO can be isolated in the filtrate. Alternatively, RuBisCO concentrates can be decolorized by incubating the solution with a FPX66 (Dow Chemicals) resin packed in a column or batch mode. The slurry is incubated for 30 minutes and then the liquid is separated from the resin. The colorants can bind to the resin and RuBisCO can be collected in the column flow-through.

In some embodiments, the isolated protein can be purified and decolorized as described in Example 3. See also "Methods for Extracting and Purifying Native Proteins" filed on October 1, 2014, U.S. Ser. No. 62/058,211.

In some embodiments, a decolorized isolated plant protein can provide an increased shelf-life stability to the red color of the meat replica as compared to a corresponding meat replica including an isolated plant protein without decolorization. In some embodiments, the decolorized protein lead to an improved flavor profile of the meat replica as compared to that observed in a meat replica with the corresponding isolated plant protein without decolorization.

Heme-containing or other proteins also can be recombinantly produced using polypeptide expression techniques (e.g., heterologous expression techniques using bacterial cells, insect cells, fungal cells such as yeast, plant cells such as tobacco, soybean, or *Arabidopsis*, or mammalian cells). For example, leghemoglobin can be recombinantly produced in *E. coli* or *Pichia pastoris* as described in Example 1. In some cases, standard polypeptide synthesis techniques (e.g., liquid-phase polypeptide synthesis techniques or solid-phase polypeptide synthesis techniques) can be used to produce heme-containing proteins synthetically. In some cases, *in vitro* transcription-translation techniques can be used to produce heme-containing proteins.

In some embodiments, the meat replicas described herein are substantially or entirely composed of ingredients derived from non-animal sources, e.g., plant, fungal, or

microbial-based sources. In some embodiments, a meat replica may include one or more animal-based products. For example, a meat replica can be made from a combination of plant-based and animal-based sources.

Making the Meat Replica

A meat dough can be prepared by mixing an isolated plant protein and an optional edible fibrous component, an optional flavoring agent, and optional non-animal fat, and adding an aqueous component such as water or a broth to the mixture and kneading or otherwise mixing, manually or mechanically, to form a dough. The aqueous component can be heated before adding to the mixture of plant protein and fibrous component. Once the meat dough is formed, the meat dough is heated (e.g., steamed or boiled) to a temperature ranging from 150°F to 250°F (e.g., 160°F to 240°F, 170°F to 230°F, 180°F to 220°F, or 190°F to 212°F). For example, a meat dough can be steamed by placing in a rice cooker, steam cabinet, or tunnel steamer. A meat dough can be heated by applying dry heat, for example, by placing in a bread maker or oven, or by immersing in hot water or broth. Boiling in broth can improve the meat dough flavor because beneficial flavors and off-flavor masking agents can be absorbed into the dough. Texture properties may also be modulated by choice of the cooking method.

As used herein, the term "isolated plant protein" indicates that the plant protein (e.g., a heme-containing protein, wheat gluten, dehydrin protein, an albumin, a globulin, conglycinin, glycinin, or a zein, or mixtures thereof) or plant protein fraction (e.g., a 7S fraction) has been separated from other components of the source material (e.g., other animal, plant, fungal, algal, or bacterial proteins), such that the protein or protein fraction is at least 2% (e.g., at least 5%, 10%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99%) free, by dry weight, of the other components of the source material. For example, wheat gluten can be used alone or in combination with one or more other proteins (e.g., dehydrins). Dehydrins can be particularly useful for enhancing the juiciness and texture in the ground meat replicas. In some embodiments, the meat replica can be formulated to be gluten free, and, for

example, a blend of maize starch, tapioca flour, rice flour, and guar gum can be substituted for the wheat gluten in the meat dough.

The edible fibrous component can be a plant fiber, an extruded mixture of isolated plant proteins (e.g., wheat gluten or other isolated plant protein, such as glutelins, albumins, legumins, vicillins, convicillins, glycinins and protein isolates such as from any seed or bean, including soy, pea, lentil, etc.), or a solution-spun protein fiber. In some embodiments, the solution-spun protein fiber is a prolamin solution-spun protein fiber. The prolamin can be from any plant source (e.g., corn or pea) and can include zein, prolamin, kafirin, secalin, hordein, or avenin. The texture of the ground meat product (e.g., meat patty) depends on properties of the edible fibrous component such as fibrousness and tensile strength. As described herein, the extruded mixture of isolated plant proteins or solution spun protein fibers can be referred to as connective tissue replicas and the fibrousness and tensile strength of the connective tissue replicas can be controlled by co-variation of extrusion parameters such as temperature, throughput, and die size. For example, combinations of lower extrusion temperatures, medium/low throughputs and smaller dies favor production of highly fibrous tissues with low tensile strength, while higher extrusion temperatures, higher throughputs and larger dies favor production of low fibrousness tissue replicas with very high tensile strengths.

The fibrousness and tensile strength of connective tissue replicas also can be modulated by changing the composition of the extrusion mixture. For example, by increasing the ratio of isolated plant protein (e.g., soy protein such as conglycinin) to wheat gluten to 3:1 w/w, and simultaneously decreasing water content in the extrusion mixture to 50%, a connective tissue replica with thinner fibers and larger tensile strength can be made.

The texture of a meat dough also can be modified by adding cream of tartar to the preparation. For example, meat dough preparations containing cream of tartar may be more cohesive, with a form factor after grinding that is similar to ground beef, such that it is readily shaped. Cream of tartar can be added between 0.05% and 2.5% (e.g., 0.5%).

The appearance of the ground meat replica can be modulated by shredding the edible fibrous component into pieces of the desired size and shape. In some

embodiments, edible fibrous component can be shredded using commercial shredders, e.g., a Cuisineart chopper/grinder, UM 12 with a dull blade attachment, Comitrol shredder (Urschel Laboratories, Indiana) or a similar shredder. The size of the fibers can be adjusted to imitate the fibrous appearance of meat by the type of shredder, choice of blade, and screen type, and adjusting the time of shredding.

In other embodiments, the edible fibrous component can be separated into fibers by carding, using hand-held carders or carding machines, for example, Pat Green carder. By varying the size and spacing of pins on the carding drums, the size of the fibers can be adjusted to imitate the fibrous appearance of meat.

In other embodiments, the edible fibrous component can be separated into fibers by pushing it through rollers (for example, a KITCHENAID® pasta attachment), followed by gentle shredding using, for example, a dull blade on a UM 12 machine. By varying the number of rollers and the spacing between the rollers, the size of the fibers can be adjusted to imitate the fibrous appearance of meat.

The fibrousness, tensile strength, and appearance of the connective tissue replicas can be tailored to imitate specific ground meat products (e.g., ground beef or different cuts of beef that can be ground).

In some embodiments, the edible fibrous component includes soluble or insoluble plant fibers. For example, plant fibers from carrot, bamboo, pea, broccoli, potato, sweet potato, corn, whole grains, alfalfa, kale, celery, celery root, parsley, cabbage, zucchini, green beans, kidney beans, black beans, red beans, white beans, beets, cauliflower, nuts, apple skins, oats, wheat, or psyllium, or a mixture thereof, can be used as the edible fibrous component.

In some embodiments, the edible fibrous component can include compounds that prevent development of off-flavors during the extrusion process. High temperature and low moisture conditions to which the extrusion mixture is exposed during the extrusion process lead to formation of compounds associated with grainy, woody, nutty, rubbery and other off-flavors. Including certain classes of compounds such as antioxidants or carotenoids can help reduce the formation of off-flavor compounds. For example, the

extruded mixture can include canthaxanthin to prevent development of grainy off-flavors. Carotenoids can be about 0% to about 1% by weight of the edible fibrous component.

In some embodiments, meat doughs are formed using roughly equal proportions of isolated plant protein and edible fibrous component. It will be appreciated that the ratio can be varied as desired to tailor the properties of the end product.

In some embodiments, a broth such as a flavored broth can be used in the meat dough. For example, a meat dough can be formed using roughly equal proportions of isolated plant protein and a broth.

In some embodiments, a flavor broth includes flavor mixtures created by pre-reacting (cooking) flavor precursors before adding into the meat dough. Flavor precursor molecules or compositions can be added to a pre-reaction mixture in purified form and/or can be derived from ingredients in the uncooked meat dough that contain and/or are enriched with one or more of the particular flavor precursors or compositions, including, for example, coconut oil, cysteine, glucose, ribose, thiamine, algal oil, lactic acid, and or yeast extract. The resultant flavor and/or aroma profile can be modulated by the type and concentration of the flavor precursors, the pH of the reaction, the length of cooking, the temperature of cooking, the type and amount of iron complex (e.g., an iron containing protein, a heme cofactor such as a heme-containing protein, or ferrous chlorophyllin) or iron salt (iron gluconate), the temperature of the reaction, and the amount of water activity in the product, among other factors. The flavor broth can contain non-animal products (e.g., plant) or it can be a combination of animal and non-animal based precursors (e.g., lard). The flavor broth can bring flavors into the consumable food product that result in taste and smell of beef, bacon, pork, lamb, goat, turkey, duck, deer, yak, bison, chicken or desirable meat flavor.

In some embodiments, a flavored broth can be made by combining an iron complex (e.g., an isolated heme-containing protein) and/or an iron salt (e.g., iron gluconate, iron chloride, oxalate, nitrate, citrate, ascorbate, ferrous sulfate, ferric pyrophosphate, or any other aqueous soluble salt) with one or more flavor precursors and a fat (e.g., a non-animal-based fat), and heating the mixture to obtain a flavored broth containing one or more flavor compounds. Suitable flavor precursors include sugars,

sugar alcohols, sugar derivatives, free fatty acids, triglycerides, alpha-hydroxy acids, dicarboxylic acids, amino acids and derivatives thereof, nucleosides, nucleotides, vitamins, peptides, phospholipids, lecithin, pyrazine, creatine, pyrophosphate and organic molecules. For example, sugars, sugar alcohols, sugar acids, and sugar derivatives can include glucose, fructose, ribose, sucrose, arabinose, glucose-6-phosphate, fructose-6-phosphate, fructose 1,6-diphosphate, inositol, maltose, mannose, glycerol, molasses, maltodextrin, glycogen, galactose, lactose, ribitol, gluconic acid, glucuronic acid, amylose, amylopectin, or xylose. Free fatty acids can include caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, alpha linolenic acid, gamma linolenic acid, arachidic acid, arachidonic acid, behenic acid, eicosapentaenoic acid, petroselinic acid or erucic acid. Triglycerides can include fatty acid esters of caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, alpha linolenic acid, gamma linolenic acid, arachidic acid, arachidonic acid, behenic acid, eicosapentaenoic acid, petroselinic acid or erucic acid. Amino acids and derivatives thereof can include cysteine, cystine, a cysteine sulfoxide, allicin, selenocysteine, methionine, isoleucine, leucine, lysine, phenylalanine, threonine, tryptophan, 5-hydroxytryptophan, valine, arginine, histidine, alanine, asparagine, aspartate, glutamate, glutamine, glycine, proline, serine, tyrosine, ornithine, carnosine, citrulline, carnitine, ornithine, theanine, and taurine. Phospholipids can include a plurality of amphipathic molecules comprising fatty acids, glycerol and polar groups. The fatty acids are selected from the group consisting of oleic acid, palmitoleic acid, palmitic acid, myristic acid, lauric acid, myristoleic acid, caproic acid, capric acid, caprylic acid, pelargonic acid, undecanoic acid, linoleic acid, 20:1 eicosanoic acid, arachidonic acid, eicosapentanoic acid, docosohexanoic acid, 18:2 conjugated linoleic acid, conjugated oleic acid, or esters of: oleic acid, palmitoleic acid, palmitic acid, myristic acid, lauric acid, myristoleic acid, caproic acid, capric acid, caprylic acid, pelargonic acid, undecanoic acid, linoleic acid, 20:1 eicosanoic acid, arachidonic acid, eicosapentanoic acid, docosohexanoic acid, 18:2 conjugated linoleic acid, or conjugated oleic acid, or glycerol esters of oleic acid, palmitoleic acid, palmitic acid, myristic acid, lauric acid, myristoleic acid, caproic acid, capric acid, caprylic acid,

pelargonic acid, undecanoic acid, linoleic acid, 20:1 eicosanoic acid, arachidonic acid, eicosapentanoic acid, docosohexanoic acid, 18:2 conjugated linoleic acid, or conjugated oleic acid, or triglyceride derivatives of oleic acid, palmitoleic acid, palmitic acid, myristic acid, lauric acid, myristoleic acid, caproic acid, capric acid, caprylic acid, pelargonic acid, undecanoic acid, linoleic acid, 20:1 eicosanoic acid, arachidonic acid, eicosapentanoic acid, docosohexanoic acid, 18:2 conjugated linoleic acid, or conjugated oleic acid. In some embodiments, the polar groups are selected from the group consisting of choline, ethanolamine, serine, phosphate, glycerol-3-phosphate, inositol and inositol phosphates.

Nucleosides and nucleotides can include inosine, inosine monophosphate (IMP), guanosine, guanosine monophosphate (GMP), adenosine, or adenosine monophosphate (AMP). Vitamins can include thiamine, Vitamin B2, Vitamin B9, Vitamin C, 4-aminobenzoic acid, choline, niacin, Vitamin B8, Vitamin B12, biotin, Betaine, Vitamin A, beta carotene, Vitamin D, Vitamin B6, or Vitamin E. Acids such as acetic acid, caffeic acid, glycolic acid, aspartic acid, pantothenic acid, alpha hydroxy acids such as lactic acid or glycolic acid, tricarboxylic acids such as citric acid, or dicarboxylic acids such as succinic acid or tartaric acid. Peptides and protein hydrolysates can include glutathione, vegetable protein hydrolysates, soy protein hydrolysates, wheat protein hydrolysates, corn protein hydrolysates, yeast protein hydrolysates, algal protein hydrolysates, and meat protein hydrolysates. Extracts can include a malt extract, a yeast extract, or peptone.

For example, in some embodiments, a broth can be made by combining an iron complex (e.g., an isolated and purified heme-containing protein such as leghemoglobin) and/or an iron salt (e.g., iron gluconate, iron chloride, oxalate, nitrate, citrate, ascorbate, ferrous sulfate, ferric pyrophosphate, or any other aqueous soluble salt) with one or more flavor precursors (e.g., a precursor mix shown in Table 2 or Table 13) and a fat (e.g., a non-animal-based fat), and heating the mixture to obtain a flavored broth containing one or more flavor compounds. A non-animal fat can include plant derived oils, algal oils, or oils from bacteria or fungi. Suitable plant derived oils include coconut oil, mango oil, sunflower oil, cottonseed oil, safflower oil, rice bran oil, cocoa butter, palm kernel oil,

palm fruit oil, palm oil, soybean oil, rapeseed oil, canola oil, corn oil, sesame oil, walnut oil, almond oil, flaxseed, jojoba oil, castor, grapeseed oil, peanut oil, olive oil, borage oil, algal oil, fungal oil, black currant oil, babassu oil, shea butter, mango butter, wheat germ oil, blackcurrant oil, sea-buckhorn oil, macadamia oil, saw palmetto oil, conjugated linoleic oil, arachidonic acid enriched oil, docosahexaenoic acid (DHA) enriched oil, eicosapentaenoic acid (EPA) enriched oil, or margarine. The oils can be hydrogenated (e.g., a hydrogenated vegetable oil) or non-hydrogenated. Oil fractions such as stearin (e.g., palm stearin) or olein also can be used. For example, the non-animal fat can be coconut oil, or a combination of coconut oil and stearin. In some embodiments, the fat can contain non-animal (e.g., plant) products, or it can be a combination of animal and non-animal based precursors (e.g., lard), or exclusively animal-based fat.

In some embodiments, a flavored broth can be made by combining water, a non-animal based fat such as coconut oil, and a flavoring agent such as an acid (e.g., lactic acid), a carotenoid (e.g., lutein), or an antioxidant, and heating the mixture to make a broth.

After heating the meat dough as described above, a non-animal fat optionally containing a flavoring agent can be combined with the meat dough. Typically, the meat dough is allowed to cool (e.g., to room temperature) before combining the meat dough with the non-animal fat. The non-animal fat can be flavored by combining the non-animal fat with an iron complex or iron salt and one or more flavor precursors (described above) and heating the mixture to produce the flavor compounds. The heated mixture can be cooled so that the non-animal-based fat can solidify. One or more additional non-animal fats (e.g., algal oil), one or more masking agents (e.g., a lactone such as butyrolactone, delta-tridecalactone, gamma decalactone, delta-dodecalactone, γ-octalactone, dihydro-5-methyl 2(3H)-furanone, 4-hydroxy-2,5-dimethyl-3(2H)-furanone, 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, δ-tetradecalactone, or combinations thereof), or one or more flavoring compounds (e.g., acetoin, carotenoid, antioxidant, vegetable or fruit juice, puree, or extract) can be added before the mixture solidifies to improve the flavor of the non-animal fat. In some embodiments, a combination of 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, butyrolactone, γ-octalactone, and/or δ-tetradecalactone can be used as a

masking agent. Adding one or more lactones (e.g., at a concentration of 10^{-3} to 10^{-10}) can result in a decrease in off flavors perceived as grain, eggy, bitterness, cardboard, livery, or mushroom and increase desired flavors such as creamy, buttery, caramelized, fatty, fresh, and fruity. For example, combinations of two, three, or four lactones can be used to mask properties such as bitterness. In addition, lactones also can be used at concentrations between 10^{-3} to 10^{-11} to provide desired flavors such as creamy, buttery, caramelized, fatty, fresh, fruity, tallow and meaty notes to the meat replica. Thus, lactones can be used as masking agents or as flavoring agents. Lactones can act as masking agents in other products, including, without limitation, dairy replicas such as milks, cheeses, and yogurts, or protein supplements such as protein bars and protein powders. Combinations of lactones can provide a unique flavor profile important in creating meat flavors (e.g., fatty tallow and sweet aromatics) in a food product such as a meat replica or providing a beef flavor to a non-beef food product. The meat replicas improve in overall liking and meatiness rating when lactones are added to the product. In some embodiments, for example, a combination of butyrolactone, delta-tetradecalactone, and 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone can be used to provide a meaty flavor. Lactones can be added to vegetable oil to make the fat taste more like animal fat and have an increase in perception of mouth coating. The lactones also can be added to increase the sweetness of the product without a change in the sugar content. It is to be noted that agents such as lactones and carotenoids can be used to flavor food replicas (e.g., plant-based food replicas), including meat or cheese replicas, and also can be used to alter the flavor of food products such as meats and cheeses (e.g., to increase meat or cheese flavors).

In some embodiments, carotenoids such as β-carotene, zeaxanthin, lutein, trans-β-apo-8'-carotenal, lycopene, and canthaxanthin can be used to control the creation of desirable flavors and prevent undesirable flavors from being created in food products such as plant based food products (e.g., meat replicas described herein). Carotenoids can be used to reduce off plant flavors in other food products, including dairy replicas. It was found that each type of carotenoid had different properties in creating desirable flavors and controlling off flavors. See, Examples 18 and 26. The carotenoids can increase

sweet and fatty notes that improve meat replicas when added between 0.00001% and 0.1%.

Carotenoids can be added to the meat replica by adding them into the flavor emulsion or the flavor broth. The carotenoids can be added before or after cooking. The carotenoids can be added between 0.00001% and 0.1%. When the carotenoids are added before cooking, they can act as a substrate in the reaction flavor mixtures creating the flavors before their addition into a meat replica. The carotenoids also change the pathway for other flavors being generated by acting as antioxidant. With the addition of carotenoids, the flavor emulsion can have improved flavor quality; there is a decrease in off oxidized notes (waxy, fishy, painty), decrease in other off notes (earthy, mushroom, grainy, beany), and an increase in sweet, fatty, meaty, and fresh flavors. Each carotenoid has different resulting flavor profiles. For example, adding lycopene to the flavor emulsion before cooking results in a bland flavor, whereas β-carotene is very flavorful with added fatty and meaty notes compared to the control. The flavor profile of adding the carotenoids before cooking has a large effect on the flavor profile. When adding carotenoids after cooking, there can still be beneficial effects especially in terms of decreasing off flavor generated with storage. Other flavor precursor molecules in the flavor emulsion or flavor broth have an impact on the effect of the carotenoids. The resultant flavor and/or aroma profile can be modulated by the type and concentration of the flavor precursors, the pH of the reaction, the length of cooking, the temperature of cooking, the type and amount of iron complex (e.g., a heme cofactor such as a heme-containing protein, or ferrous chlorophyllin) or iron salt (iron gluconate), the temperature of the reaction, and the amount of water activity in the product, among other factors, all of which change how the carotenoids change the flavor profile. Particular examples include how carotenoids can reduce or prevent the creation of flavor compounds generated in plant oils, particularly when there is metal in the oil source. Carotenoids, when added to flavor emulsions with fat and oils that have poly unsaturated fatty acids like linoleic, gamma linoleic, DHA, and EPA, can prevent off fishy, painty, and vegetable flavor notes and facilitate the generation of meatiness, and sweet notes.

Particularly carotenoids can reduce grainy, woody, earthy, mushroom, planty and oxidized notes. Carotenoids can be added to different parts of plant-based products to have different impact. Carotenoids can reduce or prevent the creation off flavor compounds generated in wheat flours including wheat gluten. For example, lutein can be added to raw meat dough and reduce overall flavor intensity, reduce grain, woody, and oxidized notes in the cooked meat dough and in the final product. These changes in flavor character is supported by reduction in particular flavor compounds as seen by SPME Gas chromatography–mass spectrometry (GC-MS) in some cases and in other cases there is no change in flavor compounds but an observed reduction in the grain character, suggesting that carotenoids act by changing chemical reactions that are taking place and by masking particular flavors. Additionally, carotenoid added to the meat dough resulted in the samples being described as more fatty and sweeter than the control without carotenoids. The main compounds that decreased with lutein included oxidized flavor compound like alcohols and aldehydes, including (Z)-2-nonenal, (E,E)-2,4-nonadienal, and 1-penten-3-ol; additionally, sulfur compounds were decreased with lutein, including methanethiol, 2-acetylthiazole, and dimethyl sulfide; many of these compounds were also described as grainy and oxidized notes by trained flavor scientist by Gas Chromatography-Olfactometry (GCO).

Antioxidants such as epigallocatechin gallate (EGCG) also can be used to reduce off flavors in food products such as plant-based products (e.g., a meat replica). Antioxidants like EGCG, which is found in (and can be purified from) green tea extracts, can be added from 0.0001% and 0.1%. Antioxidants including EGCG also can be added to meat dough and change the flavor profile of both the cooked meat dough and the consumer products created from the dough. The EGCG decreases the overall flavor of the dough and particular decreases off flavors like grainy, and oxidized flavors as described by trained flavor scientist and confirmed using GCMS.

Vegetables or fruits (juice, purees, or extracts) can be added to meat replicas to increase the perceived meat flavor (e.g., the meatiness) and likeability of the products, as well as increase the perceived fattiness and fat mouth coating. Additionally, they can cause tasters to have an increase in salivation when eating the products, leading to an

increase in perceived juiciness in meat replicas. The type of meat flavors that the vegetable or fruit enhances depends on the type and processing. Examples include added tallow fatty notes from cucumber and melons that are enhanced with cooking; added sweet aromatics, char meat, and savory notes from honeydew; added sweet aromatics, and freshness from pineapple and, added savory, browned meat flavor from tomato.

The vegetable or fruit can be added to meat replicates in the form of juices, purees, extracts created from pressing, juicing, stream distillate, pressure distillation, solvent assisted flavor extraction, or other methods. The vegetable or fruit can be uncooked or untreated, or can be cooked or otherwise treated (e.g., by pasteurization or by enzyme inactivation) to denature proteins (e.g., lipoxygenase). The flavor profiles – both meatiness and amount of off notes, including green or vegetable notes of the fruit or vegetable – can change depending on cooking or other treatment, and depending on the amount and process of cooking or other treatment. Many of the flavors in fruit and vegetable extracts, purees, and juices are created by enzymes. These enzymes can create desirable or undesirable flavors, and the desired flavor depends upon the application for the extracts and juices. Selection of the appropriate type of fruit or vegetable and treatment allows the creation of flavors appropriate for meat replicas. In addition, during processing it can be desirable to deactivate enzymes that can cause off flavors. A particular enzyme that can generate off flavors in the extracts when added to meat replicates is lipoxygenase, which is particularly active in the skin of fruits and vegetables. Disruption of the skin can increase lipoxygenase activity. Therefore, enzyme inactivation before cutting the skin of the fruit or vegetable can help to reduce off flavors. In the generation of fruit and vegetable extracts, purees, or juices, the enzymes can be deactivated by heating above 60°C, high pressure pasteurization, or enzyme inhibition. In some embodiments, for example, lipoxygenase can be inhibited by the addition of inhibitors such as epigallocatechin gallate (EGCG), or by addition of other redox active enzymes. In some embodiments, the whole fruit or vegetable can be cooked or treated before penetrating the skin or cooking can occur after cutting of the product. The cooking or other treatment can be rapid (minutes) or long (hours). When cooking is used, the temperature can be slightly elevated from room temperature to under pressure above

120°C. For example, the fruit or vegetable can be cooked at a temperature of 60-100°C (e.g., 70-80°C, 80-90°C, or 90-100°C). The process can include blending, straining, and or pressing. The seeds can be removed in some cases or the seeds can remain.

For example, cucumber puree added to a meat replica can provide additional fatty tallow flavor but can also bring green vegetable notes along. When the fruit is cooked first, there is a decrease in a few compounds including but not limited to 2-nonenal and 2,6-nonadienal that are responsible for the green, and strong cucumber notes. Additionally, there is an increase in buttery, fatty, and tallow flavors, which could come from an increase in the concentration of lactones as seen by SPME GC-MS. The cooking of tomatoes also enhances the meaty notes while decreasing the green and tomatoes flavors.

The fruits or vegetables flavor liquids can be added to different components of the products, for example added to the meat dough before cooking, added to the fat emulsion after or before cooking, added to a gelled matrix, added to the fully assembled product, or added to the unreacted flavor broth. The extract can be added from 0.0001% for extracts to up to 10% for purees and juices.

Acids such as lactic acid can be added to the meat dough to lower the pH and change the flavor reactions that occur with cooking and processing. Beef has a pH of around 5.5; to achieve meat dough at pH 5.5 additional acidity is needed. Lactic acid brings along a desirable fresh, sourness like that seen in beef.

In other embodiments, the non-animal fat can include an isolated plant protein. For example, an emulsion can be made by combining a plant derived oil, algal oil, or oil from bacteria or fungi and an optional flavor agent with an aqueous solution of an isolated plant protein (e.g., conglycinin from soy), then homogenizing the mixture using, for example, a high-speed homogenizer and heating it for a short period of time, for example, 5 min at 90°C. Physical properties of the emulsion, such as melting temperature, firmness, brittleness, color can be modulated by using different types of isolated proteins, changing the protein concentration, oil-to-water ratio, speed of homogenization, heating temperature and heating time. For example, emulsions with a high oil-to-water ratio and low protein concentration are more brittle and melt easier,

while emulsions with lower oil-to-water ratio and a higher protein concentration are softer, less brittle, and more sticky, and melt at higher temperatures.

In some embodiments, an emulsion can be made by combining a plant derived oil, algal oil, or oil from bacteria or fungi and an optional flavoring agent with an aqueous solution of isolated proteins (for example, soy conglycinin) having a pH >10 (for example, pH 12) with, for example, sodium hydroxide. Agitation, stirring or homogenization of this mixture leads to the formation of an emulsion. After the emulsion is formed, the pH can be adjusted to neutral or an acidic pH by adding, for example, hydrochloric or lactic acid. Physical properties of these emulsions can be controlled by changing protein type, protein concentration, pH level at the time of homogenization, speed of homogenization and oil-to-water ratio.

In other embodiments, an emulsion can be made by mixing a plant derived oil, algal oil, or oil from bacteria or fungi, an aqueous solution of salt and flavoring agents (e.g., flavor precursors), and emulsifiers. For example, mono/di-glycerides, lecithins, phospholipids, Tween surfactants, sodium stearoyl lactylate, or DATEM (diacetyl tartaric acid ester of monoglyceride) can be used as emulsifiers. Physical properties of these emulsions can be controlled by changing emulsifier type and concentration, speed of homogenization and oil-to-water ratio.

The solidified, optionally flavor-infused and/or protein containing fat can be combined with the meat dough, and the mixture of the meat dough and non-animal fat can be broken into smaller pieces, e.g., by chopping, grinding, cutting, mincing, shearing, or tearing. In some embodiments, shearing can be applied to the dough while heating, resulting in a dough that firms up and eventually breaks into pieces during the cooking process. Accordingly, a separate step for breaking into pieces would not be necessary.

A carbohydrate-based gel and an optional binding agent can be added to the dough-fat mixture. The carbohydrate-based gels also are useful for developing the texture of the meat replica and providing juiciness to the final product without making it soggy. Typically, carbohydrate-based gels that have a melting temperature between about 45°C and about 85°C are used. Non-limiting examples of suitable carbohydrate-