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Review

Recombinants proteins for industrial uses: utilization of *Pichia pastoris* expression system

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Abstract

The innovation in industrial process with impact in the efficient production is the major challenge for actual industry. A high numerous of enzymes are utilized in at different level of process; the search for new alternatives with better characteristic has become a field of study of great interest, the recombinant protein achievement in a different host system is an alternative widely assessed for production of this. The microorganism *Pichia pastoris* has been used like a successful expression system in diverse areas, improved the yield and extraction-recovery of the product expressed. The reported of diverse authors in the production of enzymes with different application in industry is varied, in this review the different industry areas and the characteristic of the enzymes produced are detailed.

Key words: recombinant protein, protein production, yeast, industrial applications.

Recombinant Protein for Industrial Use

The production of protein for industrial use started in the end of century XIX, the industry searched new component for improve the efficiency of the process or searching alternatives to make it a most cost effective process; the development of recombinant DNA technology and the progress made in the optimization of the bioprocess with recombinant organisms offers a wide variety of alternatives in the production of proteins for use in industrial processes with new and/or better properties.

The expression systems based of recombinant proteins in yeast have proven to be an efficient and economical source of proteins of industrial interest higher eukaryotes and / or academic (Buckholz and Gleeson, 1991) becoming one of the most frequently used alternative for the production of proteins a high scale.

Pichia pastoris Like an Expression System

The use of the methylotrophic yeast, *Pichia pastoris*, as a cellular host for the expression of recombinant proteins has become increasing popular in recent times. *P. pastoris* is easier to genetically manipulate and culture than mammalian cells and can be grown to high cell densities. Equally important, *P. pastoris* is also a eukaryote microorganism, and thereby provides the potential for producing soluble, correctly folded recombinant proteins that have undergone all the post-translational modifications required for functionality. Additionally, linearized foreign DNA can be inserted in high efficiency via homologous recombination procedures to generate stable cell lines whilst expression vectors can be readily prepared that allow multiple copies of the target protein, multimeric proteins with different subunit structures, or alternatively the target protein and its cognate binding partners, to be expressed (Daly *et al.*, 2004).

Recombinant protein production in the yeast strain *Pichia pastoris* has several advantages over other eukaryotic and prokaryotic expression systems: (i) rapid growth rate, coupled with ease of high cell-density fermentation; (ii) high levels of productivity in an almost protein-free medium; (iii) elimination of endotoxin and bacteriophage con-

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tamination; (iv) ease of genetic manipulation of well-characterized yeast expression vectors; (v) absence of known human pathogenicity in the spectrum of lytic viruses that prey on *P. pastoris*; (vi) diverse post-translational modifications that include polypeptide folding, glycosylation, methylation, acylation, proteolytic adjustment, and targeting to subcellular compartments; and (vii) the ability to engineer secreted proteins that can be purified from growth medium without harvesting the yeast cells themselves (Li *et al.*, 2007).

Pichia pastoris has been exploited by biotechnologists for the high-level production of foreign proteins (Cregg *et al.*, 2004; Romanos *et al.*, 1992). In this review will focus in the different recombinant proteins with application in diverse industrial areas expressed in the system *P. pastoris*, highlighting the applicability and the main improvements obtained through this expression system.

Animal Feed Additive

Monogastric animals are unable to utilize phytic acids due to the low levels of phytase activity in their digestive tracts, by that an inorganic phosphate source is commonly added into the feed for the purposes of phosphorous supplementation (Torre *et al.*, 1991). The enzyme phytase catalyze the hydrolysis of phytic acid (*myo*inositol hexakisphosphate), the major storage form of phosphorous in plant seeds (Cheryan, 1980) thereby releasing inorganic phosphate, is regarded as an antinutrient factor since it forms insoluble complexes with proteins and a variety of nutritionally important metal ions such as calcium, zinc, magnesium, and iron, and decreasing the bioavailability of phourous (Torre *et al.*, 1991). Microbial phytases available in the market have problem in thermoestability and degradation suffers in stomach digestion; becoming not feasible for addition in feed rations like additive. An alternative arises from *P. pastoris* for obtaining protein from prokaryotes or eukaryotes microorganisms and with high yield of enzyme.

Phytase from *Asperguillus fumigates*

In the investigation realized by Rodriguez *et al.* (Rodriguez *et al.*, 2000) the analysis was focused on the differences presented in the recombinant protein obtained from *Pichia pastoris vs.* those obtained in other expression systems. The gene r-Afp expressed in *P. pastoris* in pPICZαA vector, obtained yield of 729 mg of purified protein per liter of culture, with a specific activity after purification of 43 U/mg at pH 5.5 of protein has a comparatively higher specific phytase activity than those reported values of the enzyme produced by *A. niger, H. polymorpha,* and *S. cerevisiae* which varied between 23.4 to 28.1 U/mg protein. The remarkable resistance of the *A. fumigates* phytase to heat inactivation is most appealing characteristic (Pasamontes *et al.*, 1997). In assay for temperature stability was conserved at 65 °C around of 57% and for 90 °C around of 40%. Another interesting observation in this study is the relatively high resistance of r-Afp to pepsin. Given the ratios of pepsin/phytase (w/w) from 0.001 to 0.1, r-Afp remained essentially intact after 2 h of incubation at 37 °C. On the other hand, the enzyme was degradable by trypsin depending on the ratios. While r-Afp was partially digested from the ratio of 0.001 to 0.02, the enzyme was fully degraded at the ratio of 0.1; suggests this results that r-Afp has different susceptibilities to proteolysis of pepsin and trypsin.

Phytase from *Escherichia Coli*

In this study executed by Chen *et al.* (2004) was evaluated and compared the production of recombinant protein in flask scale and high cell-density fermentation, the *appA* gene cloned in pPICZαA vector of *P. pastoris* registered phytase activity level was enhanced from 118 to 204 U/mL at the flask scale and 1880-4946 U/mL for high cell-density fermentation, this high yield would thus suggest that phytase production by *P. pastoris* was both economical and feasible. The most important result in this study is focusing in modified media for enhances the induction of recombinant protein; the phytase production was increased when a modified mBMMHY medium was used. The mBMMHY medium was modified from BMMY medium by replacing peptone and YNB with histidine and the YE concentration was reduced from 1 to 0.1%. The phytase activity in mBMMHY was 204 U/mL after an induction period of 96 h, virtually twice the level that it achieved when induction was conducted in BMMY or FBSH 118 and 123 U/mL respectively after 96 h induction. The protein concentration of cells induced in mBMMHY medium reached 6.4 g/L of culture supernatant and the phytase activity was noted to be 4946 U/mL after an induction period of 192 h.

Gelatin industry

Gelatin is in essence, denatured collagen and is prepared by hot acid or alkaline extraction of animal tissues such as bones and hides; is a well-known biopolymer and has a long history of use, mainly as a gelling agent in food. Certain characteristics limited their applications, the extraction procedure results in chemically modified gelatin peptides covering a wide range of molecular weights (Asghar and Henrickson, 1982) and traditional gelatin has a high gelling temperature due to its high content of helix-stabilizing hydroxyprolines. This can be undesirable for low temperature applications (Saddler and Horsey, 1987).

Production of non-gelling (*i.e.* non-hydroxylated) gelatin-like proteins in *Escherichia coli* has been reported. It involves the expression of synthetic genes constructed from repeating Gly-Xaa-Yaa encoding units (Gardner *et al.*, 1992). Many problems arise concerning the instability of these highly repetitive genes (Capello, 1990). Probably, native gelatin sequences derived from collagen genes are more stable than synthetic gelatin-like sequences, because

there is greater variation in amino acid usage and less repetitiveness (Fahnestock and Bedzyk, 1997).

Non-gellins gellatin

In report, generated by Werten *et al.* (1999) the production of high extracellular non-gelling gelatin in pPIC9 and a multicopy pPIC9K expression vectors of *Pichia pastoris* was described. A 0.7 kb rat denominated *COL3A1* cDNA fragment, encoding a 21 kDa gelatin and a 1.0 kb mouse denominated *COL1A1* cDNA fragment encoding a 28 kDa gelatin (Col1a1-1) and a 1.8 kb mouse denominated *COL1A1* cDNA fragment encoding a 53 kDa gelatin (Col1a1-2) were cloned. Different strategies were used to reduce the proteolytic degradation of this extremely vulnerable unfolded protein, the best results was obtained from a transformant bearing approximately 15 copies of the pCOL3A1 vector produced up to 14.8 g gelatin/liter extracellular medium and the problems of proteolytic degradation could be minimized despite the unfolded structure of gelatin trough modifications of pH fermentation and supplement the fermentation medium with casamino acids; proteolytic cleavage at specific mono-arginylic sites, by a putative Kex2-like protease, could be

successfully abolished by site-directed mutagenesis of these sites.

Wastewater Treatment and Bleaching

Laccases are blue copper-containing phenol oxidases that are widely distributed in plants and certain fungi (Alcalde, 2007). Have been ascribed diverse biological functions in different organism; the potential applications include (i) delignification and biobleaching of pulp (Bourbonnais *et al.*, 1997) (ii) treatment of wastewater from industrial plants (Bergbauer *et al.*, 1991) (iii) enzymatic modification of fibers and dye-bleaching in the textile and dye industries (Abadulla *et al.*, 2000). A possible advantage with *P. pastoris* compared to many filamentous fungi is that it does not produce cellulolytic enzymes and laccase produced in this host could, therefore, potentially be applied directly in the pulp and paper industry without any purification.

Laccase from *Trametes versicolor*

The work realized by Hong *et al.* (Hong *et al.*, 2002) utilized the gene laccase *lcc*l and was cloned into *P. pastoris* in pHIL-D2 expression vector. Was employed the BMM medium, and the highest laccase activity registred was 3.3 U/mL, which was observed after 3 days of induction; after that it declined to 1.6 U/mL. The decrease in laccase activity during the late period in shake-flask cultivation might be due to the low pH. The temperature is an important parameter for optimization of laccase expression in yeast systems (Cassland and Jonsson, 1999), the results confirmed that cultivation at low temperature in shake-flask improved laccase activity 3.3 U/mL at 30 °C and 11.5 U/mL at 20 °C.When fermentor cultivations was used the pH was kept at 5.0 and in the protease assay higher proteolytic activity was found at pH 5.0 than at pH 8.5, a previous investigation indicated that proteolytic activity played an important role in laccase expression by *P. pastoris* because the protease deficient SMD 1168 strain performed better than GS115. In the study a positive correlation was established between the amount of methanol consumed and the quantity of protein produced was observed, however a fivefold higher volumetric laccase activity was obtained when the methanol concentration was kept at 0.5% instead of 1.0%.

Laccase from *Pleurotus sajor-caju*

Research realized by Soden *et al.* (2002) report the cloning of the full-length gene for one of these isozyme genes, namely Psc *lac4*, and on its subsequent expression in the heterologous host *P. pastoris*. The GS115 strain was utilized transformed with pPIC3.5-*lac4*, the level of Psc Lac4 expression reported here was 4 ± 85 mg/L the purified protein had an activity of 1050 U/mL, with a specific activity of 2100 units mg^{-1}. Psc Lac 4 was stable for at least 5 h between pH 6 and pH 8. Over the same period of time, the activity decreased to approximately 80%, 70% and 64% of the original value when the enzyme was incubated at pH 5, pH 3 and pH 9, respectively. At 25 °C, the pH stability of Psc Lac 4 was reduced. The optimal temperature for Psc Lac4 activity was 35 °C with ABTS as substrate. Psc Lac4 was relatively stable after 1 h at 35 °C; however, over the same period of time residual activity decreased to approximately 35% and 25% of the original value when incubated at 35 °C and 45 °C respectively, while no detectable activity was observed after 1 h at 65 °C.

Laccase from *Pycnoporus cinnabarinus* I-937

Studies realized by Otterbein *et al.* (2000) was dedicated to isolation and characterization of a cDNA corresponding to the gene *lac1* isolated from fungus *P. cinnabarinus* I-937 and its expression in the methylotrophic yeast *P. pastoris*; two distinct expression plasmids were used. Plasmid pPICZB/Lac1 and pPICZaB/Lac1 differs from the first in that it contains the native *S. cerevisiae* α-factor secretion signal upstream of the sequence of mature laccase. Expression was evaluated in baffled flasks and for pPICZαB/Lac1 construction, the laccase activity gradually reached 20 units, and the cell culture density reached a D600 of 21 after 10 days of incubation for other hand for the pPICZ/Lac1 expression vector, laccase activity reached a maximum of 12 units and the cellular density reached a D600 value of 17 after 8 days, the expression level was satisfactory 8 mg/L with the two expression vectors used, but the eventual proteolytic degradation events in the culture medium could not be regulated, the regulation of pH in the media utilized was important factor for the production of

laccase enzyme, obtaining in acidic pH an detrimental effect in the production.

Antifreeze

Antifreeze proteins (AFP) have been isolated from a number of organisms including fish, insects, plants and bacteria (Davis and Hew, 1990). Their properties include depressing freezing temperature without affecting the melting temperature (so-called thermal hysteresis), modifying ice crystal morphology, inhibiting ice crystal growth (recrystallization), enhancing cellular integrity, and reducing microbial growth (Chapsky and Rubinsky, 1997; Harding *et al.*, 1999).

The use of AFPs is focused on industry foodstuffs that involved frozen storage. However, according to their properties, AFPs are likely to be good preservatives, not only for frozen food but also for chilled food (Griffith and Ewart, 1995).

In the work realized by Loewen *et al.* (1997) describing the shake-flask expression of the cystine-rich SRAFP in Pichia obtaining secreted recombinant protein up to 5 mg/L and demonstrate that the recombinant protein has identical activity to SRAFP isolated from sea raven serum. In order to increase yields further, four different strategies were tested in 10 liter fermentation vessels, the mixed-feeding/Mut^s strategy proved to be the most efficient with SRAFP yields reaching 30 mg/L.

Lytic Enzymes

Enzymes such as cellulases, xylanases, proteases, lipases, amylases, phosphatases and pectinases are widely used in the pulp and paper, textiles, detergent, food and beverage and pharmaceutical industries (Cheng *et al.*, 1999).

The main industrial application of pectinases is the extraction and clarification of fruit and vegetable juices. Most of the microbial pectinases produced by the industry are dedicated to this purpose. Pectins are responsible for the turbidity and consistency of the juice causing an increase in their viscosity, which hinders its clarification, filtration and concentration (Alkorta *et al.*, 1998).

Polygalacturonases are the pectic enzymes which are most commonly used in the case of orange juice, where natural pectin esterases are present, pectin is only partially methylated. In the process of orange juice extraction, pectinases can be added at the end of the pulp wash extraction to reduce viscosity or, preferably, at the end of the first finisher. This leads to higher yield in juice, a better extraction of soluble solids and to a lower viscosity. The action of these enzymes just reduces the viscosity without attacking the insoluble pectin that maintains the stability of the cloud (Kashyap *et al.*, 2001). The endopolygalacturonase is the most substantial member of pectolytic enzymes that are utilized to degrade various pectic substances (Whitaker, 1990).

Cellulases and xylanases are active fibrolytic enzymes and have sparked interest for a number of biotechnological applications. These include development of probiotic and as feed additives for silages and total mixed rations, for saccharification of lignocellulosic residues and for production of polysaccharide-hydrolysing enzymes (Li *et al.*, 1996).

Xylan is the second most abundant biopolymer after cellulose and the major hemicellulosic polysaccharide found in plant cell walls. Xylanolytic enzymes are mainly produced by saprophytic fungi and bacteria and hydrolyze the xylan component of plant cell walls (Coughlan and Hazlewood, 1993).

Endopolygalacturonase from *Botrytis cinerea*

In investigation realized by Kars *et al.* (2005) the coding sequences of the genes Bcpg1, Bcpg2, Bcpg3, Bcpg4 and Bcpg6 were cloned in the expression vector pPIC3.5 of *P. pastoris*. Production levels of BcPGs were reproducible for individual enzymes but varied significantly between the different enzymes, ranging from 5 mg/L for BcPG1 to 1000 mg/L for BcPG3. The overall yield of pure protein of the different BcPGs was highly variable ranged from 0.5 to 500 mg per fermentation. The optimal enzyme activity using polygalacturonic acid (PGA) as substrate was observed at pH 4.2 for BcPG1, pH 4.9 for BcPG4, and pH 4.5 for BcPG2 and BcPG6. Purified BcPG3 displayed a broader pH optimum ranging from pH 3.2 to 4.5.

Endo-β-1,4-xylanase from *Aspergillus niger*

In the working realized by Berrin *et al.* (2000); the cDNA-encoding XylA was used to transform *P. pastoris* and the cells with construction obtain a secretion yields up to 50-100 mg/liter were obtained in selective medium. The specific activities of recombinant xylanase of *A. niger* were 350 ± 25 U mg/L for the one expressed in *Pichia* and 270 ± 16 U mg/L for the native xylanase over expressed. The optimum reaction temperature of both xylanases was found at 50 °C and over 70% of maximal activity between 40 and 60 °C. Around 90% activity remained after 3 h incubation at 50 °C but between 3 and 4 h of incubation at this temperature, xylanase activity dropped dramatically and decreased by more than 50%.

Conclusion

All revised investigation in this review demonstrating the major production of product when the *Pichia pastoris* expression system was utilized and when this is compared with other expression system like *E. coli, S. cereviciae* or *H. polimorpha*. Recombinant protein obtained in *P. pastoris* system exhibit one aspect that has improved is the specific activity of the enzyme produced, detecting in-

creased enzyme activity when this was assessed. Being this a highly relevant aspect because when a recombinant protein is destined to industry use, the yield of the product is an important factor for profitability of the process.

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