Journal of Biotechnology 202 (2015) 118–134

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Expression of enzymes for the usage in food and feed industry with *Pichia pastoris*

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ARTICLE INFO

Article history:
Received 12 August 2014
Received in revised form
28 December 2014
Accepted 7 January 2015
Available online 14 February 2015

Keywords:
Yeast
Pichia pastoris
Protein expression
Food and feed industry
Fructooligosaccharides
Phytase

ABSTRACT

The methylotrophic yeast *Pichia pastoris* is an established protein expression host for the production of industrial enzymes. This yeast can be grown to very high cell densities and produces high titers of recombinant protein, which can be expressed intercellularly or be secreted to the fermentation medium. *P. pastoris* offers some advantages over other established expression systems especially in protein maturation. In food and feed production many enzymatically catalyzed processes are reported and the demand for new enzymes grows continuously. For instance the unique catalytic properties of enzymes are used to improve resource efficiency, maintain quality, functionalize food, and to prevent off-flavors. This review aims to provide an overview on recent developments in heterologous production of enzymes with *P. pastoris* and their application within the food sector.

1. Recombinant proteins for industrial use

By the end of the 20th century, researchers were able to produce the first recombinant proteins for industrial use. Since then, industry has continually searched for new components to improve the benefit of this development along with its cost-efficiency. The progress in the optimisation of bioprocesses and the development of recombinant DNA technology offers a wide variety of alternatives in the production of proteins with new and better properties. Yeast has proven to be an efficient host for recombinant protein expression (Buckholz and Gleeson, 1991), and has become one of the most abundant alternatives for large-scale protein production.

2. Introduction

The methylotrophic yeast *Pichia pastoris*, currently reclassified as *Komagataella pastoris* (Kurtzman, 2009; Yamada et al., 1994, 1995), was introduced by Phillips Petroleum more than four decades ago for the commercial production of single cell protein (SCP) as an animal feed additive. It has since become a substantial expression system in biotechnological processes, especially for heterologous protein production (Kurtzman, 2009). In 1973 the price for methanol increased drastically as a consequence of the oil crisis, which made SCP production uneconomical. *P. pastoris* was developed as a protein expression system in the 1980s using the tightly regulated alcohol oxidase promoter AOX1 (Cregg et al., 1985) which provided exceptionally high levels of heterologous protein expression. The production of the plant-derived enzyme hydroxynitrile lyase at 20 g of recombinant protein per litre was the first large-scale industrial production process established in the 1990s (Hasslacher et al., 1997). The first host strains for heterologous protein expression were GS115 (Schutter et al., 2009) and *P. pastoris* DSMZ 70382 (Mattanovich et al., 2009).

For long time *P. pastoris* was not considered to be as genetically amenable as *Saccharomyces cerevisiae*. However, the publication of detailed genome sequences was a major breakthrough (Küberl et al., 2011). Since then, genetic advances including the development of a *P. pastoris* strain, which has the capacity to produce "humanized" glycoproteins, have been made. This permits the production of active recombinant erythropoietin (Hamilton et al., 2006). The most important breakthrough for the usage of the *P. pastoris* in food technology was the GRAS (generally recognized as safe) status by the Food and Drug Administration (FDA) and

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http://dx.doi.org/10.1016/j.jbiotec.2015.01.027
0168-1656/

the FDA approval of recombinant proteins (Ciofalo et al., 2006; Thompson, 2010). Today, more than 500 pharmaceutical compounds and recombinant proteins are known to be produced with *P. pastoris* (Macauley-Patrick et al., 2005a).

In the last decade, this expression system has been reviewed extensively with the focus being on genetics and its usage in drug and pharmaceutical industries (Ahmad et al., 2014; Cereghino and Cregg, 2000; Daly and Hearn, 2005; Jin et al., 2006; Macauley-Patrick et al., 2005a). In this article, we focus on recent developments in heterologous production of enzymes with *P. pastoris* for the usage in food and feed industry.

3. Comparison of the expression system *P. pastoris* with *E. coli* and *S. cerevisiae*

E. coli has been used extensively as a host for recombinant protein expression in the last four decades. Since this simple microorganism lacks the intracellular machinery to achieve post-translational modification, the application of this system with eukaryotic proteins has been problematic. Therefore, the successful expression in *E. coli* depends on the protein sequence, secondary and tertiary fold, and the functional characteristics of the recombinant protein. The inability to fold heterologous proteins correctly and perform further post-translational modifications limits the protein expression. The protein product may be obtained as insoluble, miss-folded inclusion bodies. As a result, subsequent solubilisation and re-folding are mandatory (Makrides, 1986, 1996). The reducing environment of the cytoplasm and inadequate chaperones can lead to incorrect folding (Bardwell, 1994; Cole, 1996; Li et al., 2001; White et al., 1994). For these reasons *E. coli* is not generally suitable for the expression of proteins containing disulphide bridges (White et al., 1994) or proteins requiring other post-translational modifications like disulphide isomerization, lipidation, glycosylation, proline cis/trans, phosphorylation or sulfation (Lueking et al., 2000). The stability of proteins expressed in *E. coli* is also affected by the presence of their amino-terminal methionine (Chaudhuri et al., 1999; Takano et al., 1999). Due to the lack of proper glycosylation of proteins in *E. coli*, the function of certain recombinant proteins can be altered (Jenkins et al., 1996; Meldgaard and Svendsen, 1994).

A difficult and time-consuming refolding of a recombinant protein can result in significant losses, lower productivities and increased manufacturing costs of the expressed protein (Tsujikawa et al., 1996; Wang et al., 2000). Proteins that cannot be expressed in *E. coli* with correct post-translational maturation have been successfully produced with the methylotrophic yeast, *P. pastoris* (King et al., 1995; Lueking et al., 2000). Further *E. coli* is unable to secrete proteins beyond its periplasm into the medium. Since *P. pastoris* has the capability to secrete the authentic protein to the medium in a soluble form, like *S. cerevisiae*, it has the capacity to produce large quantities of enzymes via economically attractive downstream processing without laborious purification.

Many of the secreted proteins of *S. cerevisiae* are not found free in the medium, but rather in the periplasmic space, what leads to problems with purification resulting in decreased product yield (Buckholz and Gleeson, 1991). There are several reasons that make *P. pastoris* a more amiable expression system. For one, the developed bioprocesses for *S. cerevisiae* can be easily applied to *P. pastoris*. Furthermore, the strong, inducible *AOX1* promoter can be used for protein production. In contrast to *S. cerevisiae*, the lower levels of host protein secretion in *P. pastoris* facilitate the isolation of the recombinant protein. The above mentioned progress in glycoengineering enables the production of further functional proteins for the food industry. For example, bulky high-mannose-type N-glycan blocks the taste-modifying activity of miraculin, a sweet tasting protein (Ito et al., 2010). Here glycoengineered *P. pastoris* can be a very suitable host for high-level production of active protein. Further information on the comparison between *P. pastoris* and *S. cerevisiae* can be found in literature (Näätsaari et al., 2012).

Due to all these reasons, *P. pastoris* expression systems offer significant advantages for the production of many heterologous eukaryotic proteins.

4. Enzymes in food technology

Food enzymes can be divided into the following categories depending on the intended use: food ingredients, food additives and processing aids. Food ingredients are enzymes added for nutritional reasons, but this is rarely the case. More commonly, enzymes are added for technical reasons. Enzymes which are still present in an active form in the end product are defined as food additives. Otherwise the proteins in the product commonly belong to the category of processing aids.

In 2008 the European Commission (EC) made the so-called Food Improvement Agents Package, defining all food enzymes with technological purposes as a separate group (Regulation (EC) No. 1332/2008), regulating food additives (Regulation (EC) No. 1333/2008), flavourings (Regulation (EC) No. 1334/2008) and a common authorization procedure (Regulation (EC) No. 1331/2008).

Although not in isolated form, enzymes have been used traditionally for dairy, baking, brewing, and winemaking for centuries. Enzymes are needed for cheese production and a wide variety of other dairy goods. For example, their application keeps bread soft and fresh longer, leads to crispy crusts, increases dough volume and can compensate for variations in flour and malt quality. Additionally, enzymes are used to lower alcohol concentration and calories in beer. In winemaking, the sulphur content can be reduced, clarity and wine colour can be maintained, flavours can be enhanced and the filterability can be improved with enzymes. They are also utilised to improve the quality, stability, clarity and yield of fruit juices. The starch and sugar industry has been revolutionized by the usage of enzyme catalysis to hydrolyse starch and to rearrange glucose into fructose. In new approaches even cellulose can be transformed into fructose by enzyme catalysis (Lee et al., 2013). Galactooligosaccharides (GOS) and fructooligosaccharides (FOS) are well-established non-digestible oligosaccharides, which provide several health benefits and have excellent technological properties that make them attractive food ingredients. FOS can be produced by the degradation of fructan using Inulinase (described below) or by transglycosylation of sucrose, while GOS can be produced from Lactose using β-galactosidase (Czermak et al., 2004; Engel et al., 2007; Gonzalez et al., 2009; Ebrahimi et al., 2010; Kovács et al., 2014).

Enzymes are highly valuable for the food and feed industry on account of their wide range of applications and cost-efficiency. In 2013 the world industrial enzyme market had a value of approximately €3.27 billion, according to the global market leader Novozymes with 47% market share (Novozymes, 2013). Further important enzyme producers are Danisco with 21% market share, DSM with 6% market share, AB Enzymes with 5% market share and BASF with 4% market share. Food and beverage enzymes make 29% of sales in enzyme business, which is outnumbered only by household care enzymes with 31%. Feed and other technical enzymes accounted for 13% of sales.

The first five of the six different enzyme classes (EC 1: oxidoreductases, EC 2: transferases, EC 3: hydrolases, EC 4: lyases, EC 5: isomerases, and EC 6: ligases) are sold commercially for food and feed production. So fare ligases are not used in food and feed production. In the European Union, approximately 260 different enzymes are available (cf. Table 4). They are isolated from fungi