Biotechnology Advances 33 (2015) 1433–1442

Contents lists available at ScienceDirect

Biotechnology Advances

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

Research review paper

Halophiles, coming stars for industrial biotechnology☆

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ARTICLE INFO

Available online 27 October 2014

Keywords:
Halophiles
Polyhydroxyalkanoates
PHB
Ectoines
Bio-surfactants
Halomonas
Industrial Biotechnology

ABSTRACT

Industrial biotechnology aims to produce chemicals, materials and biofuels to ease the challenges of shortage on petroleum. However, due to the disadvantages of bioprocesses including energy consuming sterilization, high fresh water consumption, discontinuous fermentation to avoid microbial contamination, highly expensive stainless steel fermentation facilities and competing substrates for human consumption, industrial biotechnology is less competitive compared with chemical processes. Recently, halophiles have shown promises to overcome these shortcomings. Due to their unique halophilic properties, some halophiles are able to grow in high pH and high NaCl containing medium under higher temperature, allowing fermentation processes to run contamination free under unsterile conditions and continuous way. At the same time, genetic manipulation methods have been developed for halophiles. So far, halophiles have been used to produce bioplastics polyhydroxyalkanoates (PHA), ectoines, enzymes, and bio-surfactants. Increasing effects have been made to develop halophiles into a low cost platform for bioprocessing with advantages of low energy, less fresh water consumption, low fixed capital investment, and continuous production.

Contents

1. Introduction

Industrial biotechnology has been developed substantially in the past years with an aim to partially replace petroleum based chemical industry (Chen et al., 2010; Gartland et al., 2013; Otero and Nielsen, 2010). However, bio-based products such as biochemicals, bioplastics and biofuels are still too expensive due to their high production cost compared with chemical counterparts (Philp et al., 2013). The high production cost of bioprocessing is mainly associated with the following issues. First, the price of raw materials (substrates), e.g. glucose from hydrolysis of starch, has increased very fast (Tanadchangsaeng and Yu, 2012). Second, bioprocessing requires large amount of fresh water, making water shortage even worse (Chen, 2012). Third, most of the fermentation processes (bioprocessing) are run discontinuously to avoid microbial contamination, which results in low production efficiency. Fourth, the energy intensive sterilization of the fermentors and piping

☆ This paper is for "Biotechnology Advances" **special issue** dedicated to two recent conferences: (1) 18th Biochemical and Molecular Engineering Conference (BME XVIII) and (2) 4th International Conference on Biorefinery towards Bioenergy 2013 (ICBB 2013).

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http://dx.doi.org/10.1016/j.biotechadv.2014.10.008
0734-9750/

systems as well as the medium is a very expensive process (Wang et al., 2014). Fifth, also the investment to purchase stainless steel bioprocess facilities is heavy. Lastly, Procedures to maintain contamination free and batch processes make the bioprocessing very complicated, leading to increasing cost for biochemical production.

To make industrial biotechnology as competitive as the chemical industry, it is urgent to develop low cost bioprocessing technology. In such technology, low energy and fresh water consumptions as well as contamination free continuous fermentation processes are required in addition to low cost substrates.

Halophilic microorganisms have recently been re-discovered to possess advantages for the above mentioned desirable properties (Wang et al., 2014). Due to their unique halophilic properties, many *Halomonas* spp. can grow in high pH and high NaCl containing medium at a larger temperature range, making contamination free fermentation processes under unsterile conditions and continuous way possible. Recently, genetic manipulation methods have been developed for *Halomonas* by the authors' lab (Fu et al., 2014), allowing diversification of products from *Halomonas* spp.

This review summaries recent developments of *Halomonas* spp. as well as other halophiles and efforts made using the species for bioprocessing with advantages of low energy, less fresh water consumption, low fixed capital investment, and continuous production.

2. Biology of halophilic microorganisms

Halophiles (salt-loving) are referred to those microorganisms that require salt (NaCl) for growth, and they can be found in all three domains of life – Archaea, Bacteria and Eukarya (Quillaguamán et al., 2010). Halophiles can be found in hypersaline environments which are widely distributed in various geographical areas on earth, such as saline lakes, salt pans or salt marshes (Setati, 2010). According to the salt concentration for optimal growth, halophiles can be roughly divided into two groups, moderate and extreme halophiles (Oren, 2008; Ventosa et al., 1998). A moderate halophile grows at salt concentration of 3-15% (w/v) and can tolerate 0-25% (w/v) (Ventosa et al., 1998). A large number of phylogenetic subgroups contain many types of halophilic bacteria, most of which belongs to the family *Halomonadaceae* (class Gamma proteobacteria) (Oren, 2002).

To thrive in the hypersaline environment, halophiles have two main adaptation mechanisms to prevent NaCl from diffusing into the cells. The first mechanism is accumulation of inorganic ions (mainly KCl) for balancing osmotic pressure. This mechanism is mainly utilized by aerobic and extremely halophilic archaea and some anaerobic halophilic bacteria (Oren, 1999, 2002). In contrast, most halophilic bacteria and eukarya accumulate water soluble organic compounds of low molecular weight, which are referred to as compatible solutes or osmolytes, to maintain low intracellular salt concentration (Oren, 2008; Quillaguamán et al., 2010; Roberts, 2005). Compatible solutes can act as stabilizers for biological structures and allow the cells to adapt not only to salts but also to heat, desiccation, cold or even freezing conditions (Delgado-García et al., 2012), allowing the halophile to grow at around pH 10 and over 50 °C (Hozzein et al., 2013). Many halophilic bacteria accumulate ectoine or hydroxyectoine as the predominant compatible solutes. Other intracellular compatible solutes include amino acids, glycine betaine and other osmotic solutes accumulated in small amounts (Louis and Galinski, 1997; Vargas et al., 2008; Ventosa et al., 1998).

3. Application of halophiles for biotechnological industries

Since *Halomonas* spp. favor a condition of high salt concentration under which non-halophiles can not grow, they can be used for development of open unsterile and continuous fermentation process. Johnson et al. (2009) achieved a record of 3-year fermentation process without contamination by feeding acetate to the mixed bacterial culture. Tan et al. (2011) cultivated a *Halomonas* strain termed TD01 for 14 days in an open unsterile process for polyhydroxybutyrate (PHB) production. More recently, a halophilic bacterium termed *Halomonas campaniensis* LS21 was isolated and continuously cultured for 65 days under unsterile conditions without contamination (Yue et al., 2014).

Halophilic microorganisms, especially *Halomonas* spp., have been the candidates for production of diverse products used in various industrial fields (Table 1). Many halophiles are found to be able to accumulate polyhydroxyalkanoates (PHA), a family of biodegradable plastics (Quillaguamán et al., 2010). Ectoine and hydroxyectoine from halophiles are commercially used as protective agents for mammalian cells and for skins (Lippert and Galinski, 1992; Pastor et al., 2010). There are also growing interest in the alkaline enzymes produced by halophilic microorganisms (Setati, 2010). Moreover, many halophilic microorganisms are producers of bio-surfactants (BS) and bio-emulsifiers (BE) (Satpute et al., 2010).

3.1. PHA production by halophilic microorganisms

Polyhydroxyalkanoates (PHA) are a family of biodegradable and biocompatible polyesters accumulated by many microorganisms, PHA are developed into an industrial value chain ranging from bioplastics, biofuels, fine chemicals to medicine (Chen, 2009; Chen and Patel, 2012). PHA have diverse material properties due to over 150 monomer variations (Chen and Wu, 2005; Steinbüchel and Valentin, 1995). Among these PHA, poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) are two of the most well studied polymers and have been produced in large scale (Chen, 2009; Steinbüchel and Füchtenbush, 1998). PHB is rigid and brittle while PHBV is more flexible with wider application potentials as medical materials, films products, disposable items and packaging materials (Chen, 2009; Philip et al., 2007).

After PHA accumulation by halophiles first observed in 1972 (Kirk and Ginzburg, 1972), more and more halophiles have been exploited and found to synthesize PHA (Fernandez-Castillo et al., 1986; Han et al., 2007; Koller et al., 2007a; Legault et al., 2006). Among those PHA producing halophiles, the archaeon *Haloferax mediterranei* produced 46 wt% PHA (Lillo and Rodriguez-Valera, 1990; Rodriguez-Valera and Lillo, 1992). Further study showed that the PHA synthesized by *H. mediterranei* was a copolymer of PHBV when carbohydrates such as glucose, extruded starch or hydrolyzed whey was used as substrate (Chen et al., 2006; Don et al., 2006; Koller et al., 2007b). Several halophilic strains were also reported to synthesize PHBV from non-fatty acid carbohydrates as carbon source (Van-Thuoc et al., 2012). Han et al. (2013) described four pathways in *H. mediterranei* leading to propionyl-CoA, the precursor for 3-hydroxyvalerate (3HV) in PHBV, including citramalate/2-oxobutyrate pathway, aspartate/2-oxobutyrate pathway, the methylmalonyl-CoA pathway and 3-hydroxypropionate pathway.

The halobacterium *Halomonas boliviensis*, a representative of the family *Halomonadaceae*, tolerates salt concentration of 0-25% w/v. It grows from 0-45 °C under pH 6-11 (Quillaguamán et al., 2004). The strain utilizes carbon sources including glucose, xylose, or sucrose, and can produce PHB with high molecular weight of 1,100 kDa under its optimized condition (Quillaguamán et al., 2005, 2006, 2007, 2008).

Recently, two more *Halomonas* spp. were isolated and showed a great potential for low cost PHA production. *Halomonas* sp. TD01 grows optimally at salt concentration of 5-6% (w/v) at pH of 9.0 (Tan et al., 2011). The strain grew rapidly to over 80 g/L cell dry weight (CDW) in a lab fermentor and accumulated over 80% PHB on glucose salt medium (Tan et al., 2011). A two-stage open and continuous fermentation process was tested, in which the cultures from the first fermentor was pumped continuously to the second fermentor containing nitrogen-deficient glucose salt medium. *Halomonas* TD01 accumulated PHB up to 70 wt% CDW with a glucose to PHB conversion ratio