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**Economic Aspects of
Biopolymer Production**

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BC	bacterial cellulose
DCW	dry cell weight
DO	dissolved oxygen
EPS	exopolysaccharide
3HA	3-hydroxyalkanoate
3HB	3-hydroxybutyrate
3HHx	3-hydroxyhexanoate
3HV	3-hydroxyvalerate
HPLC	high-performance liquid chromatography
LDPE	low-density polyethylene
MCL	medium-chain-length
γ -PGA	poly- γ -glutamic acid

PHA	polyhydroxyalkanoate
SCL	short-chain-length
SSF	solid-state fermentation
TCA	tricarboxylic acid

1 Introduction

During recent years, a variety of biopolymers have become available for use in many applications that are not only compatible with human lifestyle but also are friendly to the environment. As our understanding of the biosynthesis of biopolymers and fermentation process development has advanced, it has become possible to produce an increasing number of biopolymers, in adequate quantities, and from renewable resources. In nature, biopolymers often play important roles in maintaining cell viability by conserving genetic information, by storing carbon-based macromolecules, by producing either energy or reducing power, and by defending an organism against attack from hazardous environmental factors (Steinbüchel, 2001). Biopolymers are superior to petrochemical-derived polymers in several aspects that include biocompatibility, biodegradability, and both environmental and human compatibility. Biopolymers can be classified according to the monomers that constitute them, and include various polysaccharides, polyamides (proteins and poly- γ -glutamic acid (γ -PGA)), nucleic acids (DNA and RNA), polyesters (polyhydroxyalkanoates, PHAs), polyphosphates, and polyisoprenoids (natural rubber). Today, some of these biopolymers are produced by bacterial fermentation and are used commercially in a wide range of applications such as foods, pharmaceuticals, plastics and agriculture. Not only refined carbohydrates but also agricultural and dairy byproducts can be used as substrates for the production

of these biopolymers by fermentation processes. Some important biopolymers, for example cellulose and starch, are obtained directly from plant materials. Hence, it is unnecessary to produce these by fermentation due to the higher production costs, except for certain materials such as bacterial cellulose which has been produced by fermentation and used for specialty applications. The structures and chemical compositions of biopolymers are rather complex, and this makes their chemical synthesis both inefficient and expensive. Therefore, the development of biotechnological processes is an inevitable route towards the economic production of biopolymers.

Until now, very few biopolymers have been produced on a commercial basis due to their high production costs compared with chemically synthesized polymers that possess similar material characteristics. Consequently, much effort has been devoted to the development of processes for biopolymer production by optimizing the upstream to downstream engineering strategies including the metabolic and cellular engineering of host cells, efficient fermentation and recovery processes, and post-production modification of the biopolymers obtained. In this chapter, the various strategies developed and used for the economical production of biopolymers are reviewed. Using PHA as an example, those factors are described which affect the economics of biopolymer production.

2

Historical Outline

Some important aspects in the development of biopolymer production are listed below:

- 1926 Discovery of poly-3-hydroxybutyrate (poly-(3HB)) in *Bacillus* sp.
- 1937 Discovery of poly- γ -glutamic acid (γ -PGA) excreted from *Bacillus* sp.
- 1950 Discovery of xanthan
- 1957 Cellulose synthesis in *Acetobacter xylinum*
- 1958 Development of E medium for the production of γ -PGA
- 1960 Aerated and agitated cultivation of bacterial cellulose (BC)
- 1976 Commercial production of pullulan
- 1983 Discovery of medium chain-length polyhydroxyalkanoate (MCL-PHA) in *Pseudomonas oleovorans*
- 1988 Cloning of poly(3HB) biosynthesis genes from *Ralstonia eutropha*
- 1989 Discovery of PHA consisting of both short chain length (SCL) and MCL monomers
- 1992 Production of poly(3HB) from cheap carbon source such as whey by recombinant *Escherichia coli*
- 1994 Screening *Aeromonas* strains accumulating poly(3HB-co-3HHx)
- 1997 Fed-batch cultivation of *Alcaligenes latus* for the production of poly(3HB) under nitrogen limitation
- 1997 Economic evaluation of processes for the production of poly(3HB) and poly(3HA) by bacterial fermentation
- 1998 Cloning of poly(3HB) biosynthesis genes from *A. latus*
- 1998 Fed-batch cultivation of recombinant *E. coli* harboring *A. latus* poly(3HB) biosynthesis genes in chemically defined media for the production of poly(3HB)

- 1999 Fed-batch cultivation of recombinant *E. coli* harboring *A. latus* poly(3HB) biosynthesis genes in chemically defined media for the production of poly(3HB-co-3HV)
- 1999 Production of poly(3HB-co-3HHx) by recombinant *Ralstonia eutropha*
- 2000 Production of poly(3HB) from whey by fed-batch cultivation of recombinant *E. coli* in lab-scale fermentor
- 2000 Development of fed-batch cultivation strategy for the production of γ -PGA
- 2000 Fed-batch cultivation of *A. hydrophila* for the production of poly(3HB-co-3HHx)
- 2001 Development of cell recycle fed-batch cultivation strategy for the production of poly(3HB) from whey by recombinant *E. coli* in lab-scale fermentor
- 2002 Production of poly(3HB) from whey by fed-batch cultivation of recombinant *E. coli* in large-scale fermentor

3

Microbial Polysaccharides

Many microorganisms produce exopolysaccharides (EPSs) as an extracellular or cell surface-attached material in the form of amorphous slime (Silman, 1990; Sutherland, 1998). In microorganisms, EPSs may be associated with virulence in some cases, and may protect cells from desiccation or from attack by bacteriophages and protozoa in other cases (Zajic, 1973). EPSs play a major structural role in the formation of biofilms, in which various prokaryotic and eukaryotic microorganisms grow while attached to solid-liquid interfaces (Catley and Whelan, 1971; Sutherland, 1998). Several of

these microbial polysaccharides are now widely accepted industrial products, while others are in various stages of development (Rogovin et al., 1965; Davidson, 1978; Catley et al., 1986; Cannon and Anderson, 1991).

At present, the major sources of polysaccharides are plants or marine algae (Kaplan, 1987), and these have been widely used in the food and pharmaceutical industries (De Vuyst et al., 1987a, b). Among the various polysaccharides, cellulose and starch are readily available from many sources in virtually all parts of the world. Microorganisms synthesize a wide range of polysaccharides, even though the actual composition is restricted to a relatively small number of monosaccharides and some other noncarbohydrate substituents such as acetate, pyruvate, succinate, and phosphate. Many microbial EPSs provide material properties that are almost identical to the gums in current use, but show improved rheological characteristics that might apply to new uses.

Microbial EPSs can be divided into homopolysaccharides and heteropolysaccharides (Yuen, 1974; Weiss and Ollis, 1980; Byrom, 1991; Joris and Vandamme, 1993; Garcia-Ochoa et al., 1995). Most homopolysaccharides are neutral glucans, while the majority of the heteropolysaccharides are polyanionic due to the presence of uronic acids. Further contributions to charge come from pyruvate ketals or succinyl half-esters. At present, very few EPSs are available commercially, but the number and their applications are gradually increasing. Some of the microbial polysaccharides such as xanthan (the EPS from *Xanthomonas campestris* pv. *campestris*) are already well established by modern biotechnology and have a sizable market (Kennedy and Bradshaw, 1984; Quinlan 1986; Garcia-Ochoa et al., 1993, 1995, 1996, 2000; Garcia-Ochoa and Gomez, 1998; Letisse et al., 2001). Others such as pullulan possesses potentially useful chemical and physical

properties, and consequently are receiving much attention from industrial areas. Microbial EPSs are promising substitutes for traditional plant polysaccharides because of their unique and superior physical properties. However, the products of microbial fermentation always compete with synthetic polysaccharides that are generally cheaper to produce. In this section, the fermentation and recovery methods of bacterial cellulose, xanthan and pullulan are reviewed.

3.1

Bacterial Cellulose (β -D-Glucan)

Cellulose (β -1,4-glucan) is one of the most abundant polymers in nature. Bacterial cellulose (BC) has unique physical properties, which differ from those of plant cellulose (Lee, 1981), and it has therefore attracted much attention as a new functional material (Shibazaki et al., 1993; Watanabe et al., 1998). BC is an exopolysaccharide produced by *Acetobacter xylinum* and by some other, mainly Gram-negative, bacterial species (Jonas and Farah, 1998). BC is excreted into the medium where it rapidly aggregates as microfibrils, yielding a surface pellicle. Since BC is highly pure and shows unusual physico-chemical characteristics, a number of industrial applications are possible (Table 1). Unfortunately, the current price of BC remains too high to make it commercially attractive for a wide range of applications.

3.1.1

BC Fermentation

One conventional method of BC production is static cultivation, whereby as it is produced it forms a pellicle. This process is not suited to large-scale production as productivity is highly dependent on the surface area of the medium (Schramm and Hestrin, 1954). In the past, the establishment of a process to

Tab. 1 Applications of bacterial cellulose (BC)

<i>Material</i>	<i>Application</i>	<i>References</i>
Temporary artificial skin Biofill®, Bioprocess®, Gengiflex®	Therapy of burns, ulcers, dental implants	Patent WO 086020
Nonwoven paper or fabric	Improvement of latex or other binders	Patent WO 08901074
Sensitive diaphragms	Stereo headphones	Cannon and Anderson (1991)
Cellulose	Immobilization of proteins, chromatographic techniques	Patent JP 07274988
Edible cellulose	Addition to food	Patent JP 07079769
Cellulose	Stabilizer of emulsions in cosmetics, food	Patent JP 08033495
Cellulose	Coating compositions	Patent US 5360723

produce BC economically has been pressing, and although both aerated and agitated cultivations have been carried out, the productivity was relatively low (Dudman, 1960). In agitated culture, the BC produced forms a pulpy suspension, but the recent development of cellulose-producing bacteria suitable for agitated cultivation (Ishikawa et al., 1995; Toyosaki et al., 1995), together with modifications in agitator design (Kouda et al., 1997), have led to improved production. Indeed, by improving the culture condition in a stirred tank fermentor (Kouda et al., 1997; Naritomi et al., 1998, 2002), a productivity of $0.115 \text{ g L}^{-1} \text{ h}^{-1}$ was obtained for BC (Toyosaki et al., 1995).

An air-lift fermentor has been used for such processes as wastewater treatment, single cell protein production and fungal cell cultivation (Onken and Weiland, 1983), because this process requires less energy to operate and is easy to scale up. Bearing in mind the high viscosity of BC, it is credible to use an air-lift fermentor for its production, especially as the BC productivity achieved was comparable with that obtained in a stirred-tank fermentor (Chao et al., 1997).

3.1.2

Culture Media

The cost of the carbon source contributes significantly to the overall production cost of BC, and using a cheaper carbon source can lower such costs, particularly in industrial-scale fermentations. Although glucose is a good carbon source for BC production, two sugar alcohols – arabitol (Oikawa et al., 1995a) and mannitol (Oikawa et al., 1995b) – have led to the production of 6.2 and 3.8 times more BC respectively than was achieved with glucose. In addition, Masaoka et al. (1993) reported that lactate had a stimulatory effect on BC production when added at 0.15% (w/v) to a medium containing fructose, soytone and yeast extract. The cost of a complex nitrogen source can also contribute significantly to the overall production cost. Yeast extract, peptone and casamino acids are good complex nitrogen sources, but are rather expensive. One of the cheapest complex nitrogen sources, corn steep liquor, has been used successfully in BC production. For example, Naritomi et al. (1998) reported that a high BC productivity ($0.62 \text{ g L}^{-1} \text{ h}^{-1}$) could be achieved in continuous fermentation using a corn steep liquor-fructose-based medium.

3.1.3

Recovery Method

In chemical terms, BCs are identical to plant cellulose, but differ in their degree of polymerization. BCs are normally of crystalline structure and their microfibrils form ribbons (Smith and Pace, 1982; Watanabe et al., 1998). BC pellicles, when floating on the surface of the medium, appear as a gelatinous layer of variable thickness (Shairi et al., 1998). For its recovery, BC is first collected and then washed with distilled water to remove the residual culture medium. In order to solubilize the proteins and small molecules, BC pellicles are boiled under a nitrogen atmosphere in 1–3% (w/v) NaOH solution. This treatment is repeated twice and is followed by extensive washing with distilled water. The NaOH digestion method is advantageous not only because NaOH is much cheaper than other chemicals used in different recovery methods, but also because much less NaOH is used to achieve similar recovery efficiency. After neutralization with acetic acid or hydrochloric acid and thorough rinsing with tap water, the BC pellicle is dried. The insoluble residue is collected by centrifugation, and washed with distilled water to neutrality.

3.2

Pullulan (α -D-Glucan)

Pullulan is an extracellular water-soluble microbial polysaccharide produced by strains of *Aureobasidium pullulans*. It is α -D-glucan in which maltotriose and a small number of maltotetraose units (1,2- α -linked) are coupled through 1,6- α -bonds to form an essentially linear polymer (Catley, 1970, 1979; Carolan et al., 1983). The molecular mass of pullulan is between 1×10^3 and 3×10^6 Da depending on the strains and cultivation conditions employed (Catley, 1970,

1979; Lacroix et al., 1985). Pullulan, being highly water-soluble, forms a viscous solution that is stable in the presence of most cations. Pullulan may be used as a coating and packaging material, and also as a molecular weight standard for polymer characterization (Lacroix et al., 1985). For example, it has been used to prepare molecular-mass standards of low polydispersity for calibrating high-performance liquid chromatography (HPLC) columns for the size-exclusion chromatography of water-soluble polymers (Buliga 1987; Tsujisaka and Mitsunashi, 1993). Pullulan can be used in the food industry, for example as an edible film and as a starch replacer in low-calorie food formulations, and also in cosmetic emulsions and other industrial and medicinal applications (Deshpande et al., 1992).

3.2.1

Pullulan Production by Fermentation

The production of pullulan by several different strains of *A. pullulans* using sucrose and glucose in shaking flask and stirred tank fermentors has been reported (Carine, 1985; Shabtai and Mukkmenev, 1995; Gibbs and Seviour, 1996; Youssef et al., 1999). Gibbs and Seviour (1996) reported that 6 g L^{-1} of pullulan could be produced when *A. pullulans* (ATCC3092) was grown in a synthetic medium in a stirred tank fermentor by maintaining the dissolved oxygen concentration at a low level during the initial phase of the fermentation. Youssef et al. (1999) reported that a maximum pullulan concentration of 31.3 g L^{-1} , pullulan productivity of 4.5 g L^{-1} per day, and sugar utilization of 100% could be achieved in batch fermentation. In this fermentation, the yield of pullulan on sugar was about 0.6 g pullulan per gram sugar. By fed-batch culture, 24.5 g L^{-1} of pullulan with a productivity of 3.5 g L^{-1} per day could be obtained by

feeding a nutrient solution containing 50 g L⁻¹ sucrose.

In another study, Shabtai and Mukkmenev (1995) reported enhanced production of pullulan in a two-stage fermentation by arresting cells which are morphologically similar to yeast. In the first stage of fermentation, starting at pH 4.5 with soybean oil as a carbon source and glutamate as a nitrogen source, a cell mass of 15 g L⁻¹ was obtained. When the soybean oil and glutamate were nearly exhausted, the cells were shifted to a production stage, in which sucrose was used as a carbon source with nitrogen limitation. A high concentration of pullulan (35 g L⁻¹) could be achieved in 50 h. The production yield of pullulan on the sugar was about 0.6 g g⁻¹. Recently, Roukas and Mantzouridou (2001) reported that a maximum pullulan concentration of 30 g L⁻¹ could be obtained in an air-lift fermentor at an aeration rate of 2 vvm (volume per volume per min). In order to achieve further savings in production costs and to maximize productivity and stability, West and Strohfus (1998) used an immobilized cell system in either agar or calcium alginate. In this way, pullulan could be produced for two cycles of 168 h. Cells immobilized in alginate permitted a higher production of pullulan, with 4.2 mg pullulan per gram cells per h during the first cycle, and 4.6 mg pullulan per gram cells per h during the second cycle.

3.2.2

Culture Media

A chemically defined medium containing sucrose and glucose has been most widely used for the production of pullulan by different strains of *A. pullulans* (Roukas and Biliaderis, 1995; Roukas and Mantzouridou, 2001). The production of pullulan from synthetic media containing molasses, peat hydrolyzates, fuel ethanol byproducts, Jeru-

salem artichoke tubers, carob pods and deproteinized whey in shake flask and stirred tank fermentors has also been reported (Gibbs and Seviour, 1996; Shabtai and Mukkmenev, 1995; Youssef et al., 1999). For example, Roukas and Biliaderis (1995) reported that 10 g L⁻¹ of pullulan could be produced when *A. pullulans* was grown in carob pod extract in a shake flask culture. Roukas (1999) also reported pullulan production by *A. pullulans* using brewery wastes in a shake flask culture. Here, the highest pullulan concentration was 11 g L⁻¹, the pullulan yield was 48.2 ± 1.5%, and sugar utilization was 99%. Barnett et al. (1999) reported that 27 g L⁻¹ of pullulan could be produced from potato starch waste obtained after potato snack production. Although the productivity of pullulan obtained with these inexpensive carbon sources was lower than that obtained with purified carbon substrates, the pullulan content was similar.

3.2.3

Recovery Method

A process for the isolation and purification of pullulan is shown in Figure 1, the main steps being centrifugation, evaporation, precipitation, ultrafiltration, and drying. As shown in Figure 1, the most common technique used for the primary isolation and purification of pullulan is precipitation using water-miscible nonsolvents such as methanol and/or ethanol. Knowledge of the mechanisms controlling phase separation is required for devising alternatives to methanol precipitation. Total precipitation of the pullulan is possible only when 2–3 volumes of methanol or ethanol are added per volume of fermentation broth (Gibbs and Seviour, 1996; Shabtai and Mukkmenev, 1995; Youssef et al., 1999). The pullulan concentration in solution also influences the volume of the precipitating reagent needed. The energy cost for alcohol precipitation without con-

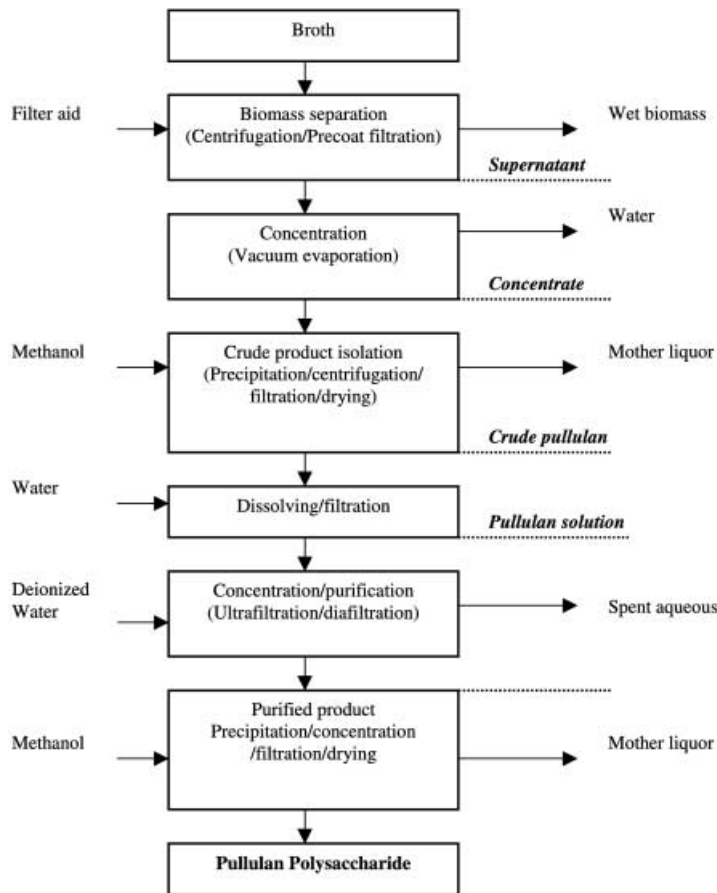


Fig. 1 Downstream process flowsheet for isolation and purification of pullulan.

sidering any alcohol loss in the process was estimated at US\$2.7 per kg pullulan processed (Flahive et al., 1994). In a different recovery method, ultrafiltration was used to concentrate pullulan from the culture broth. Under optimal conditions, and with a quoted membrane cost of US\$200 m⁻², the total operating cost for ultrafiltration of pullulan broth was about US\$1.3–1.5 per kg pullulan processed (Flahive et al., 1994).

3.3

Xanthan

Xanthan, a microbial biopolymer produced by the *Xanthomonas* species, has attracted much scientific and industrial interest since its discovery in the late 1950s at the Northern Regional Research Laboratories (NRRL) of the United States Department of Agriculture (Margaritis and Zajic, 1978; Vandamme et al., 2001). Substantial commercial production began in early 1964 (Weiss and Ollis, 1980; Kennedy and Bradshaw, 1984; De Vuyst et al., 1987b; Garcia-Ochoa and