Bioprocess Parameters of Production of Cyanobacterial Exopolysaccharide

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Published online on: 30 Jul 2019


Accessed on: 17 May 2022

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Biomass Production and Product Recovery

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BOX 3.1 SALIENT FEATURES

The exopolysaccharides (EPSs) produced by cyanobacteria are important constituents for the development of biofilm through association of microbial communities in various habitats. Discoveries in the field of cyanobacterial exopolysaccharide synthesis have opened up new opportunities for bioprocess engineers towards the production of biopolymers which is suitable for various industrial applications. Such engineering approaches are able to produce efficient exopolysaccharide as well as modified polymers exhibiting unique functional properties for specific interest. Therefore, a low-cost production system along with improving the quality of exopolysaccharide becomes economically viable, which is only possible through the development of an optimized production system. This book chapter aims to demonstrate the most important studies on the bioprocess engineering approaches of native producer for enhanced production of EPS available in literature to date. The chapter also discusses technical concerns about the application of various reactors and their optimization for high yield cyanobacterial exopolysaccharide production. In addition, progress toward purification of exopolysaccharide using suitable unit operation is highlighted extensively in this chapter.
INTRODUCTION

The marine environment is a vast and complex source of microorganisms. It envisages that one milliliter of seawater contains more than $10^5$ bacterial cells. Marine bacteria encompass many different phyla, including actinobacteria, proteobacteria, and cyanobacteria (Watson et al. 1977). Cyanobacteria are water harboring microorganisms which sustain their livelihood by gaining energy from sunlight (Bhunia et al. 2017; Sardar et al. 2018). They occur in bodies of water such as marine lakes and estuaries. The cyanobacteria play a pivotal role in controlling the biogeochemical cycle of the atmosphere (Suthers & Rissik 2009). It is evident that marine cyanobacteria yield numerous unique nutraceuticals and natural products.

The potential of cyanobacteria for the production of enormous quantity of exopolysaccharide is well recognized (De Philippis et al. 2011). The ubiquitous polysaccharides are abundantly available within microorganisms, plants, and animals such as starch, glycogen, and cellulose. They find applications as exopolysaccharide in the preparation of adhesives, biofloculants, soil booster including biosorbents, etc. (Table 3.1).

Hence, this comprehensive review encompasses and envisions the significantly influencing factors chiefly related to the yield of exopolysaccharide and its extraction from nutrient growth medium as documented in published literature.

PRODUCTION OF EXOPOLYSACCHARIDE

It has been demonstrated that controlled growth and harvesting of cyanobacterial biomass would enrich the productivity and production of value-added compounds in an economically viable manner. Bioreactors are being specifically designed for maximizing the yield of biomass and its significant products. They could be grown in open or closed systems depending on the ease of cultivation and scale-up requirements.

TABLE 3.1

The Application of Cyanobacterial Exopolysaccharide

<table>
<thead>
<tr>
<th>Cyanobacteria</th>
<th>Application of EPS</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anabaena sp. BTA992</td>
<td>Bioflocculants property</td>
<td>(Khangembam et al. 2016)</td>
</tr>
<tr>
<td>Sargassum thunbergii</td>
<td>Antitumor agent</td>
<td>(Itoh et al. 1993)</td>
</tr>
<tr>
<td>Cyanothece sp. ATCC 51142</td>
<td>Adsorbent for heavy metals, organic pollutants such as dyes and pesticides</td>
<td>(Aksu 2005; Shah et al. 2000)</td>
</tr>
<tr>
<td>Microcoleus sp.</td>
<td>Soil conditioner</td>
<td>(Mazor et al. 1996)</td>
</tr>
<tr>
<td>M. vaginatus</td>
<td>Biological soil crusts inducer</td>
<td>(Wang et al. 2009)</td>
</tr>
<tr>
<td>Arthrospira sp.</td>
<td>Thickenning agent</td>
<td>(Chentir et al. 2017; Velasco et al. 2009)</td>
</tr>
<tr>
<td>Anabaena sp. ATCC 33047</td>
<td>Biopolymer</td>
<td>(Moreno et al. 1998)</td>
</tr>
</tbody>
</table>

COMMERCIAL-SCALE ALGAE CULTIVATION

Open Cultivation Systems

In open systems, the cells are exposed directly to the atmosphere. Open systems are the oldest cultivation types and are still use widely (Figure 3.1A). In fact, the majority of commercial cyanobacterial production adopts open pond cultivation (Lee 1997). Even though many constraints of these systems hinder productivity, the open systems are still significantly cheap in comparison to closed bioreactors (Carvalho et al. 2006). Due to contamination issues, sustained production in open ponds has been successful only for a small number of organisms which thrive in extreme environments including high salinity or high pH (Parmar et al. 2011).

Shallow Ponds

The simplest systems for cultivation of cyanobacteria are shallow ponds (Richmond 2008). The production limitations, due to insufficient mixing, poor light utilization and high evaporation rates, result in a nutrient imbalance including carbon delivery which is quite inefficient. To achieve high productivity one needs to ensure easy access to water, nutrients, and sunlight for the most of the year. Moreover, the temperature should be stable, rainfall should be small, and land costs should be low. For these reasons, shallow ponds are not applicable in countries where land is expensive, and climate conditions vary to a significant extent.

Cultivation Tanks

Cultivation tanks possess a similar design to shallow ponds, but they are typically of much smaller volume, which allows better control of environmental conditions. Abyssal light utilization efficiency usually limits the productivity. Also, the gas transfer is typically insufficient, and thus possibilities for successful scale-up are minimal (Borowitzka 1999).
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Circular and Raceway Ponds

Culture reservoirs with constant circulation represent improved systems which overcome some of the constraints mentioned previously. Mixing is often secured by a rotating arm (circular ponds, Figure 3.1) or paddle wheel (raceway ponds, Figure 3.1B) (Chisti 2007). The culture depth is required to stay at the minimal level of approximately 15 cm as a prevention of flow reduction and formation of turbulence. Such depth is far from sufficient for light penetration, and typically low biomass concentrations are achieved in both raceway and circular ponds (Sheehan et al. 1998). Circular basins are slightly more expensive constructions and have significantly higher energy requirements for mixing; however, they are adopted widely. The raceway ponds are the most utilized industrial plants for outdoor cultivation.

Sloped Open Systems

Light availability is effectively managed using sloped outdoor cultivation units (Figure 3.1C) with culture depth reduced to approximately 1 cm. The liquid pump secures culture circulation and carbon dioxide incorporated into the system through its suction part. By connecting multiple cultivation panels, culture mixing is significantly improved, and thus the algae population can reach higher densities when compared to other open systems. With denser culture, harvesting costs can be reduced. However, even in sloped systems, the productivity is potentially limited by contamination and environmental variations, and the biomass productivity is far from the maxima achieved in closed cultivation systems (Olivieri et al. 2014).

Closed Reactor Systems

In closed systems, culture separates from the outside environment. Besides the possibility of avoiding contamination, closed systems offer broader options of cultivation conditions control, which gives them at least partial independence on geographical location. With increased cell densities, harvesting costs are further reduced. Moreover, they can be grown vertically in reactors reducing the land required for production.

However, the initial investments for reactors’ setup are much higher when compared to open systems, which is probably the main reason for the still limited commercial use of these systems. It is opined that capital investments required per liter volume of culture, as well as production costs for a kilogram of dried biomass, were the highest for closed reactor systems, in comparison with raceway ponds and even heterotrophic cultivation in a closed fermenter (Davis et al. 2011).

Tubular Reactors

Tubular reactors are made of flat transparent tubes connected by U-shape or L-shape bands to form flat loops which can be further arranged either vertically (Figure 3.1D) or horizontally (horizontal tubular reactor, Figure 3.1E). Gas exchange, as well as nutrient addition, is typically carried out in a separate vessel, with a pump used for culture circulation. Photobioreactor tubes

FIGURE 3.1 (See color insert.) A Circular pond, B Raceway pond, C Sloped open bioreactor, D Vertical tubular reactor, E Horizontal tubular reactor, F Flat panel laboratory reactor (Chisti 2007; Olivieri et al. 2014; Parmar et al. 2011; Posten 2009; Sforza et al. 2014).
transmit photosynthetically active irradiation and are reliable and stable in both mechanical and transparency properties. The tubes are usually of diameters between 10–60 mm, and length of up to several hundred meters (Posten 2009). Cyanobacteria can potentially attach to the internal tube walls. Besides the systems mentioned previously, a cylindrical airlift bioreactor can also be considered as a particular type of tubular reactor; with typically smaller volumes, vertical orientation, and mixing secured by gas injection (Cozma & Gavrilèscu 2012). In the cylindrical bioreactor with a fiber-optic light transmission system, the highest real production rates have been reported (Olivieri et al. 2014).

LABORATORY-SCALE BIOREACTORS

The most commercially successful cyanobacterial biotechnological applications start in the laboratory with screening experiments, identification of growth, and production optima or limitations, as well as hypotheses validations which all typically take place in a controlled laboratory environment. The cultivation of cyanobacteria can take place in theory in any vessel that secures a stable environment – thus, even the basic setups which utilize Erlenmeyer flasks or test tubes for cultivation are still very popular in research laboratories (Figure 3.1F). They are used to develop innocula for further scale-up in bioreactors described previously. Various designs of laboratory-scale bioreactors have been demonstrated for the cultivation of algal biomass (Sforza et al. 2014).

Optimization Strategies for Yield Improvement

The high productivity of the cyanobacteria is possible via boosting cellular production on a metabolic state by recombinant DNA technology as well as via designing the best-suited photoreactor encompassing the cultivation methods. The influencing parameters include water quality, temperature, light, pH, nourishments (macro/micro), and a suitable amount of salts as well as ions concentration including gaseous exchange. The cyanobacterial strains sustain an exceptional range of conditions ranging from subzero to an elevated temperature of around 70°C generally present in naturally occurring hot springs (Seckbach 2007). Likewise, extremophiles thrive in extreme pH, light, or salinity conditions. However, the parameters of the physical and chemical environment is to be optimized for maximized growth of biomass.

Algae Cultivation Regimes

Cyanobacteria is often cultured as batch, semi-continuous, or in continuous regime. Each of these modes have advantages and constraints. The simplest is the batch mode, where resources are finite, and cell concentration continually increases until some factor becomes limiting (typically some nutrient is exhausted). Potential products also increase their intensity in the medium over time. For growth restoration, the limiting factors need to be replenished. The batch growth is a highly dynamic process with culture density increasing as the typical sigmoid growth curve. Individual phases of batch growth can be categorized into lag, acceleration, exponential, retardation (“linear”), stationary, and decline period (Finkel 2006).

Another cultivation regime is the continuous mode, where cell density is maintained at a defined level by the constant addition of fresh cultivation medium. The medium inflow rate is proportional to culture growth rate and the culture removal rate. Typically, during the continuous cultivation, cells are maintained in an exponential phase, to reach maximal growth rates and biomass production. Variation of the continuous mode is the semi-continuous regime, where culture medium is replaced periodically within a defined period. A special type, standing between continuous and semi-continuous growth, is the “quasi-continuous” cultivation regime, with typically small dilution range, based on monitoring of specific culture parameters like turbidity. In the so-called turbidostat case, dilution is typically automatically controlled; it starts after the culture reach defined upper-density level and stops when the culture is diluted to a defined lower density level. Besides turbidostat, other quasi-continuous regimes have been developed, including pH-stat, physiostat, or luminostat.

EXTRACTION AND PURIFICATION OF EPS

The majority of the extraction techniques relate to a dissolvable liberated polysaccharide (DLP) within the nutrient media chiefly via unicellular cyanobacteria. The methods of extraction is often customized for enhanced recovery (Helm et al. 2000; Li et al. 2001). The common protocol adapted for extraction and purification of exopolysaccharide is shown in Figure 3.2.

TREATMENTS FOR THE EXTRACTION OF CELL-BOUND EPS

It is a general observation that a fraction of EPS is retained as adhered slime coat over the microalgal cell surface (Arad & Levy-Ontman 2010). Methods employing the use of formaldehyde (FA), ethylenediaminetetraacetic acid (EDTA), sodium hydroxide (NaOH), coupled with sonication, heating, cell cleansing in aqueous media, adoption of ionic resin are followed with suitable modification tailored to the needs to extract the
adhering EPS (Pierre et al. 2014; Takahashi et al. 2009). However, another research elucidated the application of FA along with glutaraldehyde (GTA) as a fixative for shielding microalgal cell during segregation of the EPS. An additional choice or route is to rinse the microalgae by applying water for further extraction of cell adhered EPS. Depending on the class of microalgae, warm water (30–95°C), range of pH, the extent of treatment modification (1–4 hr) is optimized for subjecting the biomass for release and dissolution of EPS. The small molecular mass (SMM) intracellular carbohydrate impurity can be removed from EPS extracts using overnight selective alcoholic precipitation with cold and absolute ethanol (−20°C) at the final concentration of 75% (in the mixture). This strategy led to the sole recovery of EPS of high molecular weights (insoluble ethanol fraction) since LMW (ethanol soluble fraction) did not precipitate.

**IsoLATION AND Extraction**

Several genera of cyanobacteria can produce EPSs. They include *Anabaena variabilis* (Bhatnagar et al. 2012), *Nostoc calcicola* (Singh & Das 2011), *Limnothrix redekei* PUPCCC116 (Khattar et al. 2010) and many more.

There are several species reviewed by Pengfu et al. (Li et al. 2001) about exploring the possibilities for the production of cyanobacterial EPS. The physical and chemical methods are generally used for the separation of soluble and bound EPS. Though the soluble EPS can be released from cyanobacteria through the centrifugation process, additional chemical treatment is required for separation of the bound EPS. Comte et al. (Comte et al. 2006) reported that the extraction process determines the EPS yield as well as the chemical composition of EPS. The ultrasonication, cation exchange resin, heating, high-speed centrifugation are adopted under physical process and alkaline reagents, ethylene diamine tetraacetic acid (EDTA), aldehyde solutions are some of the chemical process steps.

**Traditional Extraction Route via Alcoholic Precipitation**

Ramus (Ramus 1972) reported segregation of the “encapsulating polysaccharide” from *Porphyridium* biomass. Red microalgae produces sulfated polysaccharides dissolvable within the nutrient media. The removal of color from biomass was achieved by applying the acetone along with ethanol, further solubilization of exopolysaccharide glue from porphyridium cells was achieved using warm water. Another study also reports the use of absolute alcohol or isopropanol (Liu et al. 2015; Patel et al. 2013). The yield of polysaccharide is influenced by temperature of precipitation and polarity of alcohol. This technique robustly yields EPS. The method possesses specific merits like further recycling the alcohol via distillation to modify extremely viscid solution. Patel et al. (Patel et al. 2013) delineated the extraction and salting out of EPS from *Porphyridium cruentum* applying the alcoholic precipitation, separation via membrane method. Furthermore, they have inferred the application of diafiltration employing 300 kDa membrane as a
highly effective technique. Occasionally, an additional refinement phase is necessary to remove the undesired molecules like proteins, pigments, salts and another compounds via trichloroacetic acid modification, peripheral ultrafiltration, or precipitation by using alcohol (Li et al. 2011; Patel et al. 2013).

**Extraction Technique Applying Peripheral Ultrafiltration**

The peripheral ultrafiltration method is projected as a substitute to the traditional extraction of EPS applying ethanol precipitation (Charcosset 2006). The salting out method is studied by applying the diafiltration process to facilitate the substitution of the solvent via a new buffer (Charcosset 2006). The ultrafiltration approach significantly diminishes the generation of “sieve bar” and passes the suitable concentration of the biomolecules reasonably to the final edge of the filtration process. The efficacy of the membrane system is solely reliant on the following points, (i) the thickness and concentration of EPS; (ii) the orifice mass allocation along with the architecture of membrane, and (iii) the speed of the flow and the pressure of the transmembrane.

Li et al. (Li et al. 2011) established a precommercial extraction and refinement technique of functional biological EPS via the nutrient medium of numerous cyanobacteria along with microalgae like *Chaetoceros mueleri*, *Chlorella pyrenoidosa*, *spirulina platensis*, *Haematococcus pluvialis*, *Nostoc commune* along with *Nostoc sphaeroides*. The expertise is solely reliant on the microfiltration approach (polypropylene membrane) to separate microalgae along with a peripheral flow ultrafiltration system using a polyethersulfone membrane (MWCO, 5000 Da) to accumulate the EPS (20 to 40 times). Furthermore, adoption of two additional phases of the membrane process was incorporated by Maracati et al. (Marcati et al. 2014) to segregate polysaccharide along with B-PE from *Porphyridium* sp. accompanying polyethersulfone membrane possessing MWCOs around 300 kDa to 10k Da (Patel et al. 2013).

**Ultrasound or Microwave-Assisted Extraction Technique**

An unconventional route like ultrasound-assisted extraction (UAE) along with microwave-assisted extraction (MAE) is also recommended for the advancement of extraction of biomolecules chiefly from microalgae along with microalgae (Budarin et al. 2012; Kadam et al. 2013). The UAE approach has been efficiently applied to extract polysaccharides from *Spirulina platensis* (Kurd & Samavati 2015). Whereas MAE portrays the extraction procedure of intracellular metabolite as originated via microalgae like carotenoid from *Dunaliella tertiolecta* (Pasquet et al. 2011) or phycobiliproteins from *Porphyridium purpureum* (Juin et al. 2015).

**CONCLUSION/INFERENCES**

The exopolysaccharides from microalgae are gaining importance as sources of bioactive molecules of therapeutic importance. Several commercially available exopolysaccharides are obtained from cyanobacterial sources. Development of biotechnological processes for the production of biomass and downstream processing for the production of high-quality EPS products is being considered. However the process innovation is centric to the type of organism and conditions favoring the production of biomass and EPS. Accordingly the innovative interventions are being adopted for maximizing the yield for commercial feasibility.

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