ARTICLE DOI: 10.5504/BBEQ.2011.0115 A&EB

BIOSYNTHESIS OF AN EXOPOLYSACCHARIDE PRODUCED BY *BREVIBACILLUS THERMORUBER* 438

Nadya Radchenkova, Anna Tomova, Margarita Kambourova
Bulgarian Academy of Sciences, The Stephan Angeloff Institute of Microbiology, Sofia, Bulgaria
Correspondence to: Margarita Kambourova
E-mail: margikam@microbio.bas.bg

ABSTRACT

A thermophilic bacterial strain 438 isolated from the Rupi basin, Bulgaria, was chosen as a perspective exopolysaccharide producer. It was taxonomically identified as Brevibacillus thermoruber. Among ten different carbon sources tested, maltose in a concentration of 1.8% provided the highest polymer production. $(NH_4)_2HPO_4$ (0.1%) was chosen as the best nitrogen source. The highest polymer concentration (78.1 mg/l) was synthesized at pH 8.0 and 55 °C.

Biotechnol. & Biotechnol. Eq. 2011, **25**(4), Suppl., 77-79

Keywords: thermophilic bacteria, exopolysaccharide, *Brevibacillus thermoruber*, fermentation processes

Introduction

Microbial polysaccharides have proved to be useful industrial products which compete with plant and algal polysaccharides, although plant polysaccharides still dominate on the carbohydrate market. Exopolysaccharides (EPS) are water soluble polymers synthesized by many microorganisms (13) and could easily be prepared by short fermentation processes. They express novel chemical structures and consequently offer a number of novel properties and respectively applications. Their importance is highlighted by recent publications covering the chemistry, structure and function of EPS, their role in microbial ecology, medicine, dairy industry, biofilms and corrosion, and their applications in the field of biotechnology. Since the discovering of the first microbial polysaccharide, dextran, in 1880, the continued search for novel exopolysaccharides resulted in description of many new polymers, some of them commercially accepted and others at various stages of development. Currently a small number are produced in a large scale: xanthan, gellan, alginate. The intensive search for new microbial EPS is determined by the industrial need for more interesting emulsifying, gelling, flocculation properties and higher economical benefit in carbon source conversion into polysaccharide.

Still the producers of microbial EPS with industrial value are mesophilic microorganisms, although many of them are pathogenic. Referring to thermophilic producers, some obligate thermophilic bacteria (7, 11, 12, 14, 16, 17) as well as some hyperthermophiles, e.g. the archea *Thermococcus litoralis* and bacteria *Thermotoga maritima* (15), have been reported. Nowadays it is clear that some extremophiles have novel metabolic pathways (3) and in such a way might serve as a source of exopolysaccharides with novel properties with special attention to the physical properties similar to those of xanthan, however in the presence of salts, at higher temperatures or extreme of pH. Thermophilic producers suggest short fermentation processes, decreased viscosity of the fermentation broth, high molecular weight polymers and stable emulsions prepared with their polymers. Unlike mesophilic producers of EPS, thermophilic microorganisms suggest as a rule non-pathogenic product, appropriate for application in food industry, pharmacy and cosmetics. The main disadvantage in using thermophiles as EPS producers is low biomass accumulation and correspondingly low EPS production. The main attention in thermophilic processes is oriented to optimization of physico-chemical conditions for growth and search for cheap cultivation medium.

This paper deals with optimization of important fermentation parameters with the aim to increase the EPS yield.

Materials and Methods

EPS producer and polymer production

The thermophilic strain 438 isolated from a hot spring in the Rupi region, Southwest Bulgaria, was chosen as an EPS producer due to the mucoid consistency of its colonies in PY agar medium containing peptone (2 $g \cdot L^{-1}$), yeast extract (1 $g \cdot L^{-1}$) and agar (Difco) (20 $g \cdot L^{-1}$), pH 7.0 at 60 ºC.

Microbial growth and EPS production was tested in the following medium MSM (%): maltose (0.6), $(NH_4)_2HPO_4$ (0.1), $MgSO_4$ (0.01), yeast extract (0.01), KCl (0.02), thyamine hydrochloride (0.00001). Appropriate carbon source was chosen in MSM after replacement of maltose with different sugars (0.6%): sucrose, glucose, galactose, ribose, fructose, mannose, rhamnose, raffinose and cellobiose. Further optimization of the quantitative composition of the medium was performed by variation of the chosen sugar in the interval of 0.6 and 3.0% (0.6% step) and the nitrogen source $(NH_4)_2HPO_4$ was varied between 0.05 to 0.5% (0.1% step). Three different concentrations of the yeast extract were tested

* The authors N. Radchenkova and A. Tomova have equally contributed to the experimental work and the preparation of the current work for publication.

(0.01, 0.03 and 0.05%). The effect of pH on growth and EPS production was determined in the range of 4.5 – 9.5 with 0.5 pH steps. The influence of temperature was determined at pH 8.0 at 50, 55 and 60 ºC.

Isolation of EPS

Cells from early stationary phase were harvested by centrifugation (4000×g for 20 min) and the supernatant was used for EPS recovery. The supernatant was treated with an equal volume of cold absolute ethanol added dropwise with stirring in an ice bath, left to -18ºC overnight for complete precipitation of EPS and then centrifuged at 13000×g for 30 min. The pellet was dissolved in hot water and dialyzed against distilled water. Further the samples were tested for carbohydrate, protein and nucleic acid content. Carbohydrate analysis was performed by the phenol /sulphuric acid method (4) using glucose as standard. Protein contents was determined according to Bradford (2) with bovine serum albumin as a standard. Nucleic acid concentration was determined spectrophotometrically by reading the absorbance at 260 nm. Uronic acid levels were determined by the m-phenylphenol method (1).

Results and Discussion

16S rDNA analysis of the strain showed its affiliation to the species *Brevibacillus thermoruber* (99%). Its morphological and biochemical properties also were close to the type strain of this species.

TABLE 1

EPS synthesis by *Brevibacillus thermorber* 438 in a presence of different sugars as carbon source.

Sugars, 0.6%	Cell growth, OD_{660}	EPS, $mg \cdot L^{-1}$
D-galactose	0.19	39.09
D-maltose	0.22	29.41
sucrose	0.3	25.82
D-glucose	0.24	22.41
D-fructose	0.13	18.82
ribose	0.18	11.25
cellobiose	0.23	7.27
rhamnose	0.06	0
raffinose	0.09	0
mannose	0.04	0

Out of ten different sugars tested (**Table 1**), the strain was able to grow in MSM with seven of them as a carbohydrate source. The wide range of utilized sugars suggests further development of the processes based on the exploitation of waste byproducts with the aim to develop economically attractive medium. The highest quantities of EPS were registered in a medium with galactose (39.09 $mg \cdot L^{-1}$), maltose (29.41 $mg \cdot L^{-1}$), sucrose (25.82 $mg \cdot L^{-1}$), or glucose (22.41 $mg \cdot L^{-1}$). Our pursuit of developing a cheaper medium determined our interest in further exploitation of maltose as a carbon source, however a future processes based on whey is envisaged. The cultivation of the strain at different concentration of carbon and nitrogen sources showed that the most suitable concentration of maltose was 18 $g \cdot L^{-1}$ and of $(NH_4)_2HPO_4$ was 1 $g \cdot L^{-1}$. The stimulation of EPS synthesis by a high carbon/nitrogen ration was also observed by Sutherland (19).

Performed batch culture processes revealed that the polysaccharide synthesis started in the beginning of the exponential phase of growth (**Fig. 1**). First polymer appearance was registered only one hour after starting of the bacterial growth and increased in parallel with accumulation of biomass. Growth associated production of EPS was observed also by other authors (8). The EPS production increased with increasing cell density and reached its maximum (78.1 mg/l) after eight hours of cultivation in the early stationary phase. Short fermentation processes for EPS production by thermophiles were also previously reported (7, 10), while mesophilic processes continue several days (14, 18). This fact contributes to the economical efficiency of the thermophilic processes.

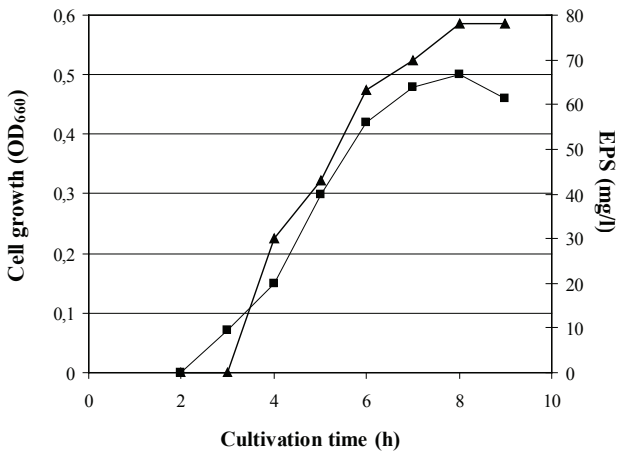

Fig. 1. Time course of growth and EPS production from *Brevibacillus thermoruber* 438 at 55 ºC, pH 8.0, 240 rpm in a defined medium. Samples were taken at 1 h intervals and assayed for growth (■) and EPS production (▲).

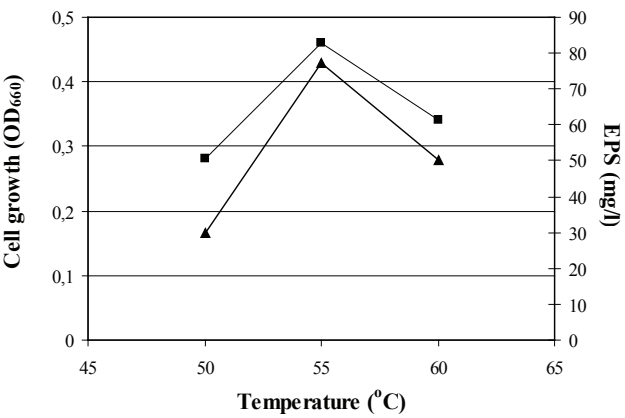

Fig. 2. Influence of temperature on growth (■) and EPS production (▲) of *Brevibacillus thermorber* 438, pH 8.0.

As thermophilic growth and EPS production are greatly influenced by physico-chemical parameters, special attention was paid on optimization of the fermentation processes. In the process of investigation of the optimal temperature for polymer synthesis, the highest quantity of EPS (78.1 mg·L^{-1}) was obtained at 55 °C (**Fig. 2**). The increase of temperature with only 5 °C resulted in decrease of the EPS production with 35% (50.53 mg·L^{-1}).

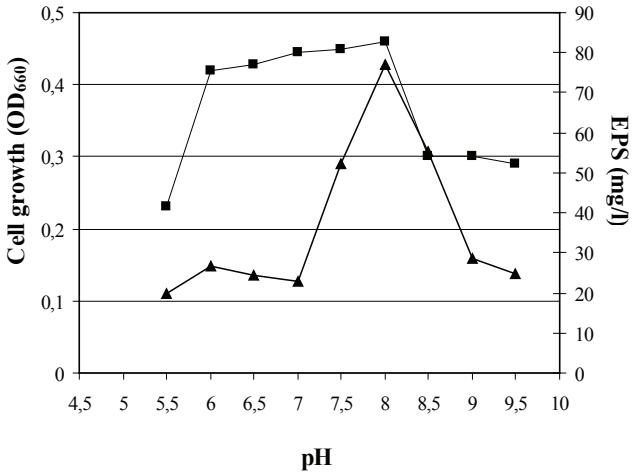

Fig. 3. Influence of pH on the growth (■) and EPS production (▲) of *Brevibacillus thermorber* 438, 55 °C.

The strain was able to grow well in a comparatively wide pH range (6-8), while the maximum of EPS production was firmly registered at pH 8.0 (**Fig. 3**). Unlike the group of lactic acid bacteria, which have also been reported as good EPS producers (5, 6, 9), thermophiles required pH to be kept around neutral for effective polymer synthesis (7, 10, 12).

Conclusions

The isolated thermophilic strain *Brevibacillus thermoruber* 438 is a perspective producer of an interesting EPS in a short fermentation process lasting eight hours. As a result of culture conditions optimization the polymer synthesis was increased almost three-fold.

Acknowledgements

This work was supported by grant BG051PO001-3.3.04/32 financed by Operational Programme Human Resources Development (2007 – 2013) and co-financed by the European Social Fund of the European Union.

REFERENCES

1. **Blumenkrantz N. and Asboe-Hansen G.** (1973) Anal. Biochem., **54**, 484-489.
2. **Bradford M.M.** (1976) Anal. Biochem., **72,** 248-254.
3. **Demirjian D., Moris-Varas F., Cassidy C.** (2001) Curr. Opin. Chem. Biol., **5**, 144-151.
4. **Dubois M., Gilles K.A., Hamilton J.K., Rebers P.A., Smith F.** (1956) Anal. Chem., **28**, 350-356.
5. **Garcia-Garibay M. and Marshall V.M.E.** (1991) J. Appl. Bacteriol., **70**, 325-328.
6. **Gorret N., Maubois J.L., Engasser J.M., Ghoul M**. (2001) J. Appl. Microbiol., **90**, 788-796.
7. **Kambourova M., Mandeva R., Dimova D., Poli A., Nicolaus B., Tommonaro G.** (2009) Carbohyd. Polym., **77**, 338-343.
8. **Kumar A.S., Mody K., Jha B.** (2007) J. Basic Microbiol., **47**, 103-117.
9. **Laws A.P., Leivers S., Chacon-Romero M., Chadha M.J.** (2009) Int. Dairy J.**, 19,** 768-771.
10. **Manca M.C., Lama L., Improta R., Eposito E., Gambacorta A., Nicolaus B.** (1996) Appl. Environ. Microbiol., **62**, 3265-3269.
11. **Moriello V., Lama L., Poli A., Gugliandolo C., Maugeri T., Gambacorta A., Nicolaus B.** (2003) J. Ind. Microbiol. Biotechnol., **30**, 95-101.
12. **Nicolaus B., Moriello V.S., Maugeri T.L., Gugliandolo C., Gambacorta A**. (2003) Recent Res. Devel. Microbiology, **7**, 197-208.
13. **Nicolaus B., Kambourova M., Oner E.T.** (2010) Environ. Technol., **31**, 1145-1158.
14. **Raguenes G., Pignet P., Gauthier G., Peres A., Christen R., Rougeaux H., Barbier G., Guezennec J.** (1996) Appl. Environ. Microbiol., **62**, 67-73.
15. **Rinker K.D. and Kelly R.M.** (2000) Biotechnol Bioeng., **69**, 537-547.
16. **Rougeaux H., Pichon R., Kervarec N., Raguénès G.H.C., Guezennec J.G.** (1996) Carbohydr. Polym., **31**, 237-242.
17. **Silipo A., Molinaro A., De Castro C., Ferrara R., Romano I., Nicolaus B., Lanzetta R., Parrilli M.** (2004) **Eur. J. Org. Chem., 24**, 5047-5054.
18. **Survase S.A., Saudagar P.S., Singhal R.S.** (2007) Bioresour. Technol., **98**, 1509-1512.
19. **Sutherland I.W.** (2001) Microbiology, **147**, 3-9.