African Journal of Biotechnology Vol. 9 (20), pp. 5454-5457, 19 October, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Exopolysaccharide production by *Bacillus subtilis* NCIM 2063, *Pseudomonas aeruginosa* NCIM 2862 and *Streptococcus mutans* MTCC 1943 using batch culture in different media

J. Francis Borgio[1]*, B. Jesvin Bency[2], S. Ramesh[3] and M. Amuthan[3]

[1]Genecity Laboratories Pvt. Ltd. 128 / 1B, First Floor, Chhatrapati House, Paud Road, Kothrud, Pune – 411038 India.
[2]Department. of Microbiology, Malankara Catholic College, Mariakiri, Kaliakavilai, 629153, Tamil Nadu, India.
[3]Post Graduate Department of Microbiology, Sri Paramakalyani College, Alwarkurichi, 627412, Tamil Nadu, India.

Accepted 21 March, 2008

Three bacterial strains, *Bacillus subtilis* NCIM 2063, *Pseudomonas aeruginosa* NCIM 2862 and *Streptococcus mutans* MTCC 1943 were examined for their exopolysaccharide (EPS) producing ability at the laboratory level. Basal salts solution (BSS), minimal salts medium (MSM), nitrogen free medium (NFM), chemically defined medium (CDM), milk medium (MLM) and sewage from different areas were used as nutrient source individually to assess EPS production by the above mentioned bacterial strains. Nitrogen free medium favoured more EPS production (Mean: 217.13, minimum: 206.000 and Maximum: 226.000). The highest EPS production was recorded in *P. aeruginosa* (226 µg ml^{-1}) grown in nitrogen free medium followed by *S. mutans* and *B. subtilis* (220 and 206 µg ml^{-1} respectively) in nitrogen free medium after 7 days of incubation at 37°C. Analysis of carbon source in sewage samples indicated the presence of reducing monosaccharides. The purified EPS was soluble only in water and was free from protein contaminants.

Key words: Exopolysaccharide, *Bacillus subtilis*, *Pseudomonas aeruginosa, Streptococcus mutans*, sewage.

INTRODUCTION

Extracellular polymeric substances of microorganisms have generated increasing attention among researchers for the last few years due to the diverse range of molecules in the exopolysaccharides (EPS) (Bhaskar and Bhosle, 2005). EPS have vital roles in a variety of processes such as formation of biofilm (Titus et al., 1995; Ramos et al., 2001; Bhaskar and Bhosle, 2005), protection of bacterial cell from desiccation (Pal et al., 1999), for maintaining primary cellular functions and antibacterial activity against predators, gelling ability, pollutant degradation kinetics (Fusconi and Godinho, 2002), bioremediation activity (Bryent, 1987) and plasma substituting capacity (Allison, 1998). EPS production in *Pseudomonas aeruginosa* reported by Huang et al. (1999), Ramphal and Pier (1985), Allison (1998), Kimmel et al. (1998), Fusconi and Godinho (2002) and Celik et al. (2007), in *Bacillus subtilis* by Ponmariappan (2003) and *Streptococcus mutans* by Vimala and Lalithakumari (2003) has been used in this study. These polysaccharides play important roles in many biological processes, and they can function as the virulence determinants in the pathogens (Peng et al., 2008).

EPS production is generally favoured by high carbon and low nitrogen ratio in the medium (Kimmel et al., 1998). Previously, several reports (Titus et al., 1995; Allison, 1998; Kimmel et al., 1998; Pal et al., 1999; Perty et al., 2000; Fusconi, and Godinho, 2002; Vimala and Lalithakumari, 2003; Peng et al., 2008) were available related to production media, but most of the researchers concentrated only on a particular medium. So it has become essential to compare the media for the highest

*Corresponding author. E-mail: borgiomicro@gmail.com.

Table 1. EPS production by *Bacillus subtilis* NCIM 2063, *Pseudomonas aeruginosa* NCIM 2862 and *Streptococcus mutans* MTCC 1943 in different media.

Tested organism	Concentration of EPS (µg ml^{-1})						
	BBS	MSM	CDM	NFM	MLM	SS1	SS2
B. subtilis NCIM 2063	146	128	168	206	18	18	10
P. aeruginosa NCIM 2862	142	204	208	226	26	12	16
S. mutans MTCC 1943	128	192	184	220	14	10	12
Control	0	0	0	0	0	4	2

BBS = Basal salts solution; MSM = Minimal salts medium; CDM = Chemically defined medium; NFM = Nitrogen free medium; MLM = Mike medium; SS 1 = Sewage sample 1; SS 2 = Sewage sample 2.

production. The present study was focused to optimize the production of EPS using different media to understand the influence of nutrients availability and growth media for EPS production. Wastewaters from different areas were also tested for their ability to serve as an energy and nutrient sources for the production of EPS by the selected microbial strains.

MATERIALS AND METHODS

Bacterial strains

Cultures of *Bacillus subtilis* NCIM 2063, and *Pseudomonas aeruginosa* NCIM 2862 were obtained from NCIM, Pune, India, and *Streptococcus mutans* MTCC 1943 from the Institute of Microbial Technology, Chandigarh, India. The strains were routinely sub-cultured and maintained in nutrient broth and were stored at 4°C in nutrient agar slants as stock cultures.

Experimental design

24 h cultures of the bacterial strain in respective media were inoculated (inoculums of 3% v/v) into 25 ml of sterilized basal salt solution (Titus et al., 1995), minimal salt medium (Vimala and Lalithakumari, 2003), nitrogen free medium (Pal et al., 1999), milk medium (De Vuyst et al., 1998), chemically defined medium (Pertry et al., 2000) and sewage water samples 1 and 2 in separate conical flasks. They were incubated for seven days at 37°C under rotatory shaker (25 rpm). Triplicates were maintained in each medium for each bacterium. Uninoculated control was also maintained for each medium. The sewage samples 1 and 2 were collected from sewage canal of Sri Paramakalyani hostel and domestic sewage canal of Mutharaman Kovil Street, Alwarkurichi, Tamil Nadu, respectively. pH value was measured and the carbohydrate source of sewage for EPS production was analysed as per the scheme of Jayaraman (1996).

Isolation, quantification and purification of EPS

After seven days of incubation at 37°C, 5 ml aliquots of the culture were centrifuged at 10,000 rpm at room temperature for 10 min. The supernatant was then filtered using 0.4 µm filters to remove the unsedimented bacterial cells. 25 µl aliquots of cell free supernatant were used for quantifying the EPS in terms of total carbohydrate using phenol sulphuric acid method (Titus et al., 1995). To the cell free supernatant, twice the volume of ice cold methanol was added, incubated overnight at 4°C and centrifuged at 10,000 rpm at room temperature for 10 min to collect the crude EPS. Crude EPS was purified as per the standard procedures (Titus et al., 1995; Vimala and Lalithakumari, 2003).

Characterization of EPS solubility and protein availability

In order to find the solubility of EPS in different solvents, small quantities of dried EPS pellet were taken in different eppendroffs to which 2 ml of solvents such as water, acetone, chloroform, ethanol, methanol and benzene were added separately, mixed thoroughly using a vortex mixture and observed for pellet formation. The availability of protein fraction in the purified and dried EPS pellet was checked by Lowry et al. (1951) method.

Statistical analysis

Results were expressed as mean and the data were analysed with descriptive statistics and compared using correlation analysis ($p < 0.05$). STATISTICA/w 5.0 software was used to perform the correlation and descriptive statistics.

RESULTS AND DISCUSSION

Table 1 exhibits EPS production during the growth period of *B. subtilis*, *P. aeruginosa* and *S. mutans* using different chosen media. Table 2 shows the descriptive statistics of EPS production by *B. subtilis*, *P. aeruginosa* and *S. mutans* in different media. The variations in the growth of different strains in different media were analysed and the results are summarized as follows. The highest EPS production was observed in *P. aeruginosa* (226 µg ml^{-1}), followed by *S. mutans* (220 µg ml^{-1}) and *B. subtilis* (206 µg ml^{-1}) grown in nitrogen free medium at 37°C for 7 days. The production on nitrogen free medium at 37°C for 7 days was significantly high ($p < 0.05$) (Table 2). The present study favours the findings of Pal et al. (1999) who reported that nitrogen free medium had stimulating effect on EPS production by microbial strains.

The lowest EPS production was recorded in *B. subtilis* (10 µg ml^{-1}) in SS2 media, *P. aeruginosa* (12 µg ml^{-1}) in SS1 media and *S. mutans* (10 µg ml^{-1}) in SS1. Considerable amount of EPS production was noted in all the tested strains when grown on basal salts solution (BSS), minimal salts medium (MSM), chemically defined medium

Table 2. Descriptive statistics of EPS production by *Bacillus subtilis* NCIM 2063, *P. aeruginosa* NCIM 2862 and *S. mutans* MTCC 1943 in different media.

Media	Valid N	Mean	Minimum	Maximum	Std. Dev
BBS	416	139.0962	128.0000	146.0000	7.59140
MSM	416	173.6346	128.0000	204.0000	33.94370
CDM	416	186.5769	168.0000	208.0000	16.74799
NFM	416	217.1346*	206.0000*	226.0000*	8.54647
MLM	416	19.5000	14.0000	26.0000	4.95765
SS1	416	13.4904	10.0000	18.0000	3.41646
SS2	416	12.6635	10.0000	16.0000	2.53785

*Highest discriptive index.
BBS = Basal salts solution; MSM = Minimal salts medium; CDM = Chemically defined medium; NFM = Nitrogen free medium; MLM = Mike medium; SS 1 = Sewage sample 1; SS 2 = Sewage sample 2.

(CDM) (Table 1). These three media were already used for EPS production by Titus et al. (1995), Vimala and Lalithakumari (2003), Petry et al. (2000) and Celik et al. (2007).

pH of the sewage samples 1 and 2 were found to be 4 ± 0.5 and 8 ± 0.5, respectively. The presence of reducing monosaccharides in the sewage samples were also established by method adopted by Jayaraman (1996).

The bacterial strains tested in the present investigation utilized the reducing monosaccharides in the sewage and produced EPS. Among the sewage sample, maximum amount of EPS was recorded in sewage sample 1 inoculated with *B. subtilis* (18 µg ml^{-1}), followed by *P. aeruginosa* (16 µg ml^{-1}) in sewage sample 2. The results of the present study favour the findings of Dave et al. (1995) who reported the presence of EPS in the wastewater treatment plant. These results are same as the findings of Fusconi and Godinho (2002); they assessed the production of EPS and their role in sewage treatment. The present study has added some more information showing that the availability of carbohydrate content in sewage can be utilized by bioremediating bacterial population to clean up the environment from hazard pollutants. It has already been known that EPS are potentially of great importance in sewage treatment processes for the removal of toxic heavy metal pollutants (Allison, 1998).

The purified EPS was soluble only in water and insoluble in other solvents such as acetone, chloroform and benzene. They were mixed thoroughly and left undisturbed, pellet formation was observed in all the solvents except water. It is the observed that the purified EPS did not contain any protein fractions. Similar result was reported by Vimala and Lalithakumari (2003).

The present comparative analysis clearly indicated that NFM is the best nurishment-containing medium for EPS production by all the three bacterial strains. Even though, quantification of EPS from the sewage indicated that their quantity was low and insignificant ($p < 0.05$), the modified sewage might be used as a nutrient for EPS production. Further studies are needed to analyse the chemical and physical characteristics of EPS produced by different strains using different media and to scale-up their production using fermenters.

ACKNOWLEDGEMENTS

JFB thanks Dr. K. Sahayaraj, Director, CPRC, St. Xavier's College (autonomous), Palayamkottai, Dr. R. Gowrisanker, P. G. Dept. of Microbiology, SPK College, Alwarkurichi for their valuable comments. JFB also sincerely thank the Secretary and Principal of Sri Paramakalyani College, Alwarkurichi for providing laboratory facilities.

REFERENCES

Allison AG (1998). Exopolysaccharide production in bacterial biofilm. Biofilm. J. 3(2): 1-19.

Bhaskar PV, Bhosle, NB (2005). Microbial extracellular polymeric substances in marine biogeochemical processes. Curr. Sci. 88(1): 45-53.

Bryent RS (1987). Potential uses of microorganisms in petroleum recovery technology. Proc. Okla. Acad. 67: 97-104.

Celik YC, Aslim B, Beyatli Y (2007). Characterization and Production of The Exopolysaccharide (EPS) from *Pseudomonas aeruginosa* G1 and *Pseudomonas putida*G12 Strains. Carbohydrate Polymers (Article in Press) (http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6TFD-4R644MV-5&_user=10&_rdoc=1&_fmt=&_orig=search&_sort=d&view=c&_acct=C000050221&_version=1&_urlVersion=0&_userid=10&md5=5a5ef1dd6d6572162d3e188f24effb00)

Dave H, Ramakrishna C, Desai JD (1996). Production of polyhydroxybutyrate by petrochemical activated sludge and *Bacillus* sp. IPCB 403. Indian J. Exp. Biol. 34: 216-219.

De Vuyst L, Vanderveken F, Van de Ven S, Deegest B (1998). Production by and isolation of exopolysaccharide from *Streptococcus thermophilus* grown in a milk medium and evidence for their growth-associated biosynthesis. J. Appl. Microbiol. 84: 1059-1068.

Fusconi R, Godinho MJL (2002). Screening for exopolysaccharide – producing bacteria from sub-tropical polluted groundwater. Braz. J. Biol. 62(2): 363-369.

Huang CT, Stewart PS (1999). Reduction of polysaccharide production in *Pseudomonas aeruginosa* biofilm by bismuth dimereaprol (Bis BAL) treatment. J. Antimicrob. Chemother. 44(5): 601-605.

Jayaraman J (1996). Laboratory manual in biochemistry – First Edition. New Age International Limited Publishers - New Delhi, India, pp. 49-60.

Kimmel SA, Roberts RF, Ziegler GR (1998). Optimization of exopolysaccharide production by *Lactobacillus delbrueckii* subsp. *bugaricus* RR grown in a semi defined medium. Appl. Environ. Microbiol. 64(2): 659-664.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Follin- phenol reagent. J Biol. Chem. 193: 265-275.

Pal S, Manna A, Paul AK (1999). Production of poly (β-hydroxy butyric acid) and exopolysacharide by *Azotobacter beijerinckii* WDN - 01. World J. Microbiol. Biotech. 15:15-21.

Peng GW, Hongjie G, Wen Y, Song, Jing K (2008). Current Understanding on Biosynthesis of Microbial Polysaccharides. Curr. Topics Med. Chem. 8(2): 141-151.

Petry S, Furlan S, Crepeau MJ, Cerning J, Desmazeand M (2000). Factors affecting exocellular polysaccharide production by *Lactobacillus delbrueckii* subsp. *bulgaricus* grown in a chemically defined, medium. Appl. Environ. Microbiol. 66(8): 3427-3431.

Ponmariappan S (2003). Corrosion control of mild steel by bacterial biofilms. Ph.D. dissertation, Manonmaniam Sundaranar University, Tirunelveli, India.

Ramos A, Boels IC, de Vos WM, Santos H (2001). Relationship between glycolysis and exopolysacharide biosynthesis in *Lactococcus lactis*. Appl. Environ. Microbiol. 67(1): 33-41.

Ramphal R, Pier GB (1984). Role of *Pseudomonas aeruginosa* mucoid exopolysaccharide in adherence to tracheal cells. Infect. Immun. 47(1): 1-4.

Titus S, Gasnkar N, Srivastava KB, Karande AA (1995). Exopolymer production by a fouling marine bacterium *Pseudomonas alcaligenes*. Indian J. Mar. Sci. 24: 45-48.

Vimala P, Lalithakumari D (2003). Characterization of exopolysaccharide (EPS) produced by *Leuconostoc* sp. V 41. Asian J. Microbiol. Biotechnol. Environ. Sci. 5(2): 161-165.