Advances in Biological Research 5 (2): 71-76, 2011
ISSN 1992-0067

Quantification and Characterization of Exopolysaccharides from *Bacillus subtilis* (MTCC 121)

P. Vijayabaskar, S. Babinastarlin, T. Shankar,
T. Sivakumar and K.T.K. Anandapandian

Department of Microbiology Post Graduate Research,
Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi- 626 124, Tamil Nadu, India

Abstract: Exopolysaccharides (EPS) are high molecular weight polymers which are long chain composed of sugar residues and secreted by microorganisms into the surrounding environment. Bacterial EPS as a complex mixture of macro molecular poly electrolytes including polysaccharides, proteins and nucleic acids, each comprising variable molecular mass and structural properties. *Bacillus subtilis* (MTCC 121) was inoculated in EPS basal and malt medium which was maintained in 72hrs at 37°C, the dry weight of the fractionated products was found to be 1.58 ± 0.13 mg/100ml and 0.82 ± 0.10 mg/100ml in basal and malt medium, respectively. From the results, it was concluded that EPS extract was higher in EPS basal medium than malt medium. The physiochemical characterization of EPS was studied and the structure was confirmed by the FT-IR, GC-MS and HPLC analysis.

Key words: *B. subtilis* · Exopolysaccharides · Physiochemical characterization · FT- IR · GC-MS · HPLC

INTRODUCTION

Exopolysaccharides are often found in the surrounding as the outer most structures of both prokaryotic and eukaryotic microbial cells. They may be closely associated with the cell in the form of discrete capsules or else excreted as slime unattached to the cell surface. EPS exist in a wide variety of unique and often complex chemical structures and they are believed to provide self protection against anti microbial substances [1].

Exopolysaccharides (EPS) produced by Lactic acid bacteria possess the possibility of replacing stabilizer and thickeners currently produced commercially by non-food grade bacteria [2]. It has been suggested that a potential controlling factor in EPS biosynthesis is the availability of sugar nucleotides which are necessary for the construction of the polymers [3]. It was found that bacterial EPS are not consumed as an energy source by the producing bacteria, but are released to protect the producer organisms under starvation conditions and also at extreme pH and temperature conditions [4]. Microorganisms belonging to *Bacillus* sp. have been known to produce extra cellular polysaccharides such as cellulose [5].

During the past 50 years a considerable number of bacterial EPS have been described, but few have achieved great commercial success due either to their being unable to offer better properties than those already on the market or to difficulties in finding new practical applications [6]. In this present study, we have quantified and characterized the exopolysaccharides produced by *B. subtilis* (MTCC 121).

MATERIALS AND METHODS

Culture Conditions: *Bacillus subtilis* (MTCC 121) culture was maintained on nutrient agar plates. It was sub cultured and slants were inoculated and maintained at 28°C for 24 hrs.

Exopolysaccharides Production from *Bacillus Subtilis* (MTCC 121): Experiments were done using 250 ml flasks each containing 100 ml of basal and malt medium inoculated with the bacterial culture. The basic medium contained EPS basal medium: Glucose-10gm, Yeast

Corresponding Author: P. Vijayabaskar, Department of Microbiology PG Research,
Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi - 626 124, Tamil Nadu, INDIA,
Mob: +91 9994019069, Fax No : 04562 254970 E-mail: baski_bos@yahoo.co.in.

extract-3gm, Malt extract-3gm, Peptone-5gm, $MgSo_4.7\ H_20$-1gm, KH_2PO_4-0.3gm and 10mg of vitamin B1 incorporated at 28°C with initial pH 5.2. Malt medium: Malt extract-40g, Peptone-5g, Distilled water -1000ml, pH -7. 24 hrs cultures were inoculated into the EPS basal medium and malt medium. It was then incubated at 37°C for 72 hrs [7].

Bacterial Eps Extraction and Quantification: Samples from flasks were separated and concentrated to small volumes [8]. The EPS was then precipitated from the supernatant by addition of equal volume of alcohol. The mixture were agitated during addition of alcohol to prevent local high concentration of the precipitate and left over night at 4°C before centrifuged at 7000 rpm for 20 min. After centrifugation, the precipitate was collected in Petri plates and dried at 60°C. EPS was extracted according to the method followed by Ohno *et al*. [9].

Estimation of Carbohydrate and Protein Content of Crude EPS: The total carbohydrate content was estimated by phenol sulphuric acid method proposed by Dubois *et al.* [10]. The amount of protein present in *B. subtilis* extract for both EPS basal medium and malt medium was estimated by the Lowry's [11] method.

Infra Red Spectroscopy Analysis of Basal EPS: The highest concentration basal bacterial exopolysaccharides were also characterized using a Fourier transform infrared spectrophotometer. IR sepectroscopies of bacterial EPS along with a standard, dextran sulfate (DS) were tested using Perkin-Elmer FT-IR instrument, which helped to analyze different sulfate, carboxyl and hydroxyl groups of these sample molecules [12]. One part of extract was mixed with ninety nine parts of dried potassium bromide (KBr) separately and then compressed to prepare a salt disc of 3mm diameter. These discs were subjected to IR- spectra measurement in the frequency range of 400 and 4000 cm^{-1} [13].

HPLC Analysis of Basal EPS: The isolated crude potential basal EPS were analyzed with a high performance liquid chromatography (HPLC) system (Agilent 1100) equipped with Aqueous GPC start up Kit column and eluted with distilled water at a flow rate of 1.0ml/min at 20°C. The separated components were monitored by a refractive index detector. The EPS after being hydrolyzed and dissolved with methanol was analyzed for its sugar composition by HPLC. The column was calibrated with different molecular mass standard and a standard curve was then established.

GC-MS Analysis of Crude Basal EPS: The basal exopolysaccharide was hydrolyzed to monomeric units and transformed in their alditol acetates. 0.1g of crude sample was mixed with 1.25ml of 72% sulfuric acid with a glass stick and incubated for 60min at 30°C. The mixtures were diluted with 13.5ml of distilled water and incubated in boiling water bath for 4 hrs. After incubation, mixtures were cooled and 3.1ml of 32% NaOH (w/v) was added. At the end of hydrolysis, 0.2ml of sample was taken separately and 2ml of 2% sodium borohydride in dimethyl sulfoxide was added. The mixtures were then shaken well at 40°C for 90min. after which 0.2ml of glacial acetic acid was added to decompose excess of sodium borohydride. After cooling, 4 ml of acetic anhydride and 0.4 ml of 1-methylimidazole were added to the solution. The mixtures were then incubated for 10 min at room temperature and then 20 ml of distilled water was added to decompose excess of acetic anhydride. After cooling, 8ml of dichloromethane was added and the mixture was shaken vigorously for total alditol acetate extraction. The upper layer was removed and the lower phase was washed three times with 20ml of distilled water. The dichloromethane was evaporated at 40°C under vacuum and final alditol acetate residues were dissolved in 1ml of dichloromethane and stored at -20°C [14].

Alditol acetates were separated on a 30 m x 0.25mm ID x 0.25µm film thickness column DB 5ms (agilent) attached to the GC- 2010 (GCM-QP 2010) SHIMADZU chromatography equipment with a flame-ionization detector and a split injector. High purity hydrogen was used as the carrier gas at a flow rate of 1.40 ml/min. The column temperature was maintained at 200°C and 240°C respectively and 1µl sample in dichloromethane was injected through a glass-lined splitter, set at 1/90 ratio. The absorption was read between 40m/z and 800m/z.

RESULTS

The EPS in the complex was separated by dissocitation of high ionic medium. The isolated EPS form both medium were estimated at 2.54 ± 0.23 mg/100ml of fresh weight in EPS basal medium and 1.47 ± 0.17 mg/100ml of fresh weight in malt medium. After dry weight of fractionated product were found to be 1.58 ± 0.13 mg/100ml and 0.82 ± 0.10 mg/100ml in basal and malt medium, respectively (Fig. 1).

Here Carbohydrate and protein estimation was done *B. subtilis* in EPS basal medium and malt medium in which the optical density for carbohydrate was 0.91 ± 0.11 mg/100ml and 0.43 ± 0.08 mg/100ml for basal and malt

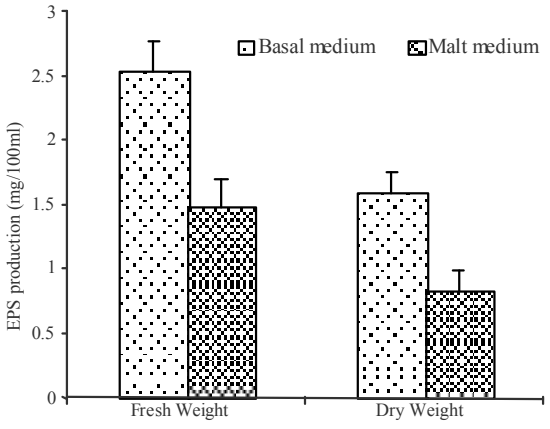

Fig. 1: Fresh and dry weight of exopolysaccharides production from *B. subtilis* (MTCC 121)

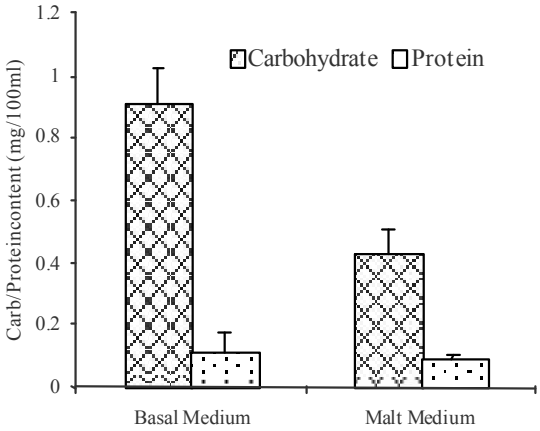

Fig. 2: Estimation of carbohydrate and protein content of bacterial exopolysaccharides

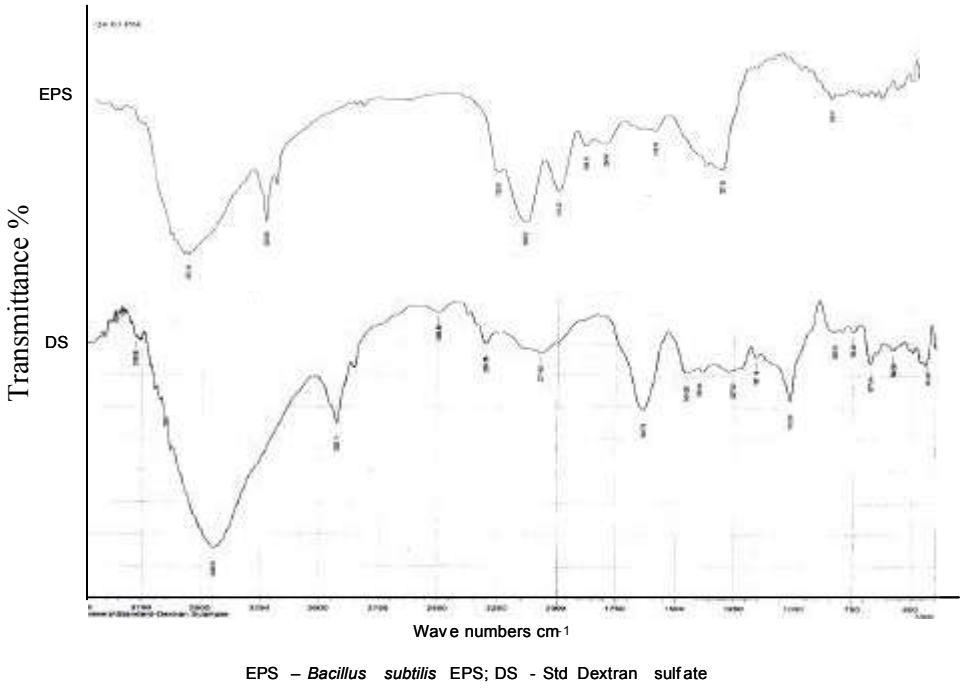

Fig. 3: FT-IR Spectrum of bound EPS in *B. subtilis* (MTCC 121)

medium, respectively. Protein was higher for *B. subtilis* in malt medium, whereas the optical density was 0.11 ± 0.07 mg/100ml and optical density for *B. subtilis* in malt medium was 0.09 ± 0.02 mg/100ml. Compared to both proteins estimation in bacteria gave higher optical density for the two media (Fig. 2).

IR spectroscopy of intact basal medium exopolysaccharides (EPS) showed the presence of hydrogen bonded compound, possible acid or amine salt. The bacterial EPS extracts revealed characteristic absorption bands of EPS as observed in the reference compound dextran sulphate (Fig. 3).

The IR spectrum of the crude polysaccharide sample showed the band at 1000-1500 cm^{-1} which is characteristic to glucan. The list of the bands at 400-950 cm^{-1} interval is present. In addition, the spectra showed bands around 1000, 1200, 1400, 1500 and 1600 cm^{-1} revealed the (1,3) -β-glucan linkages in addition to the bands in the region of 2900 and 3400 cm^{-1} chemical bands were presented.

Polysaccharides C-O-C and C-O-P was at 1037 cm^{-1}, absorption at 1000 cm^{-1} was typical for glucose in pyranose form. In the anomeric region (1000-1600 cm^{-1}) the polysaccharides exhibited the obvious characteristic absorption at 1037 cm^{-1}.

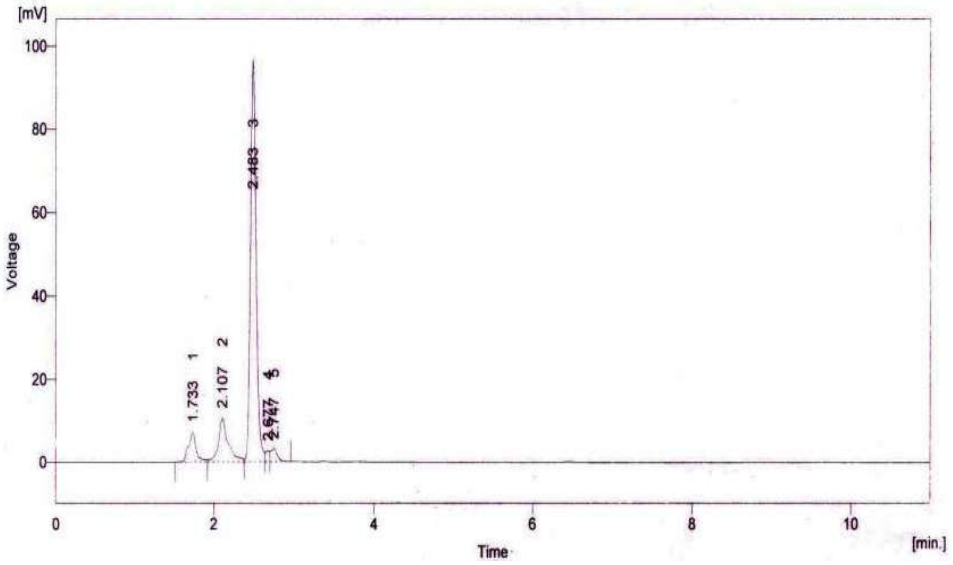

Fig. 4: HPLC analysis of exopolysaccharides in *B. subtilis* (MTCC 121)

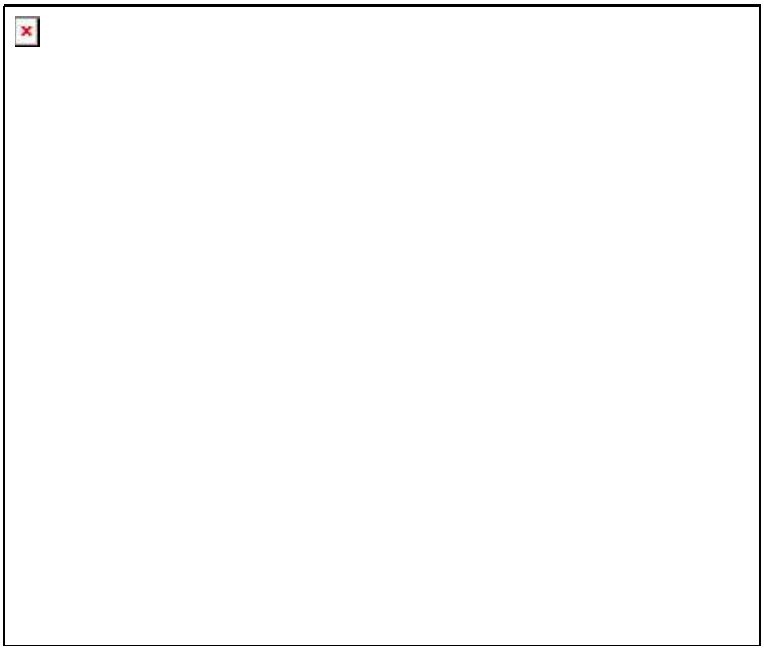

Fig. 5: GC MS analysis of exopolysaccharides in *B. subtilis*

The obtained fractions were analyzed with a high performance liquid chromatography (HPLC) system (Agilent 1100) equipped it has been confirmed with the previous reference. Here the EPS production has been quantified through HPLC, in which for EPS production from *B. subtilis* from potential EPS basal medium with the retention time as the EPS production was found to be higher in EPS basal medium. Independent peaks were identified with retention time (Fig. 4).

The fully methylated products were hydrolyzed with as it, converted into the alditol acetate and analyzed by GC-MS. Methylation analysis of the polysaccharides glucan such as 2,3,4, β-tetra-Me.Glu. and 2,3,6- tri. Me-Glu. When the oligosaccharide alditol contain hexoses. (Gal) or deoxyhexoses (Rha), the sequence of this saccharide may be determined on the basis of MS. The electron impact fragmentation patterns of the mass spectra of derived alditol acetates were prepared from the hydrolysed EPS (Fig. 5).

DISCUSSION

High level of EPS production was achieved by *B. subtilis* (MTCC 121) in basal extract medium. The usage of EPS compounds in the food industry has been intensively investigated. The novel properties of microbial exopolysaccharides such as xanthan, alginate and curdlan may improve food viscosity, hydration of products and low calories food production. It is also considered to apply the microbial EPS for food edible coating production that effectively would protect products from spoilage [15].

The filtered broth obtained from submerged fermentation contains water soluble EPS that can be recovered by precipitation with 3-4 volumes of ethanol 95%. Lee *et al*. [16] has also reported the use of 2 volumes of acetone. Some investigators dialyze the filtered broth before adding it to the ethanol solution to eliminate smaller molecules, as oligo and monosaccharides, which might interfere with the quantification of exopolysaccharides [17].

The bacterial EPS extracts gave characteristics bands for EPS. Here, carbonyl (C=O) stretching peak and OH stretching peak was at broad and the maximum peak and the band at 1000-1500 showed the presence of polysaccharide. According to Sutherland, [6] reduction of the cultivation temperature by 10°C below optimal level inhibits the EPS biosynthesis by microbial cells. However, under low temperature of the growth, environment profiles of the high productivity of extracellular polysaccharide occur by bacterial cells.

Burns *et al*. [18] reported that after few days the onset of EPS production from bacteria, its level in the culture medium declined which might be due to the secretion of β 1, 3-glucanases. Here, the *B. subtilis* excreted significant maximum quantities of EPS, when cultivation occurred under optimum growth conditions, ultimately the greatest quantity of bacterial growth where glucose was utilized as carbon source in the EPS basal medium.

In many habitats, bacteria form sessile communities known as biofilms [19], the main components of the biofilm matrix are the microbial cells products where together form a dynamic environment in which the microbial cells is organized to make use of all available nutrients.

The IR spectrum of the polymer proved the presence of carboxyl group, which may serve as binding sites for divalent cations. The carboxyl group may also work as functional moieties to generate new or modified polymer variants using different approaches like novel. The time dependent increase in the Si-O stretching vibration (1200-1000 cm^{-1}) hindered our ability to extract IR data from this region of the EPS silica spectrum symmetric phosphate stretch of nucleic acid and C-O stretching modes of sugar/sugar phosphate [20].

Here, HPLC was applied in which different independent peaks were identified and molecular mass was determined with retention time. Some papers have reported the molecular mass of different fractions from exopolysaccharides while in this study, fraction from *B. subtilis* from EPS medium was considered [21].

In case of complex EPS, neutral sugars are identified by their derivatives, alditol acetates by GC-MS [22], which was described by Hoebler *et al*. [23]. Alditol acetates are often used only to determine the ratios of monosaccharide's in polysaccharides hydrolysates [24] and in our study, the two major peaks present corresponds to 1,1, Ethanediol, diacetates, 1-Tri decenal dicholorobenzne, hexoses, deoxyhexoses for bacterial EPS. A number of small peaks are also present, but these do not necessary correspond to alditol acetate.

CONCLUSIONS

In this work, extraction of EPS from *Bacillus subtilis* (MTCC 121) and detection of the amine and carboxyl groups containing polysaccharides was carried out. These promising results can be regarded as an initiatory steps towards the utilization and modification of exopolysaccharides as future cheap sources for production of valuable drugs for antioxidant and anticancer properties.

ACKNOWLEDGEMENT

The authors are thankful to Prof. S. Baskaran, Principal and Prof. D. Prabhu, Head, PG Department of Microbiology, Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi for providing all facilities.

REFERENCES

1. Kumon, H., K. Tomoshika, T. Matunaga, M. Ogawa and H.A. Ohmori, 1994. Sandwich cup method for the penetration assay of antimicrobial agents through *Pseudomonas* exopolysaccharides. Microbiol. Immunol., 38: 615-619.
2. Welman, A.D., I.S. Maddox and R.H. Archer, 2003. Screening and selection of exopolysaccharide producing strains of *Lactobacillus* sub sp. *Bulgaricus*. J. Applied Microbiol., 95: 1200-1206.

3. Boels, I.C., R. Vavkranenburg, J. Hugenholtz, M. Kleerebezen and W.M. De Vas, 2001. Sugar catabolisim and its impact on the biosynthesis and engineering of exopolysaccharide production in lactic acid bacteria. International Dairy J., 11: 723-732.
4. Kim, H.O., J.M. Lim, J.H. Joo, S.W. Kim, H.J. Hwang, J.W. Chio and J.W. Yun, 2000. Optimization of submerged culture condition for the production of mycelial biomass and exopolysaccharides by *Agrocybe cylindracea*, Bioresour. Technol., 96: 1175-1182.
5. Hestrin, S.M., A. Aschner and A. Mager, 1994. Synthesis of cellulose by resting cells of *Acetobacter xylinum*. Nature. 159: 64-68.
6. Sutherland, J.W., 2002. Biopolymers polysaccharides from prokaryotes. In: E.J. Vandamme, S. De Baets, A. Steinbuchel. Eds., 5: 1-19.
7. Banerjee, D., M. Jana and S. Mahapatra, 2009. Production of exopolysaccharide by endophytic *Stemphylium* sp. Micologia Aplicada International. 21(2): 57-62.
8. Titus S., N. Gasnkar, K.B. Srivastava and A.A. Karande, 1995. Exopolymer production by a fouling marine bacterium *Pseudomonas alcaligenes*. Indian J. Mar. Sci., 24: 45-48.
9. Ohno, N., N. Miura, M. Nakajima and T. Yadomae, 2000. Antitumor 1-3-β-glucan from cultured fruit body of *Sparasis crispa.* Boil. Pharm. Bul., 23: 866-872.
10. Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith, 1956. Colorimetric method for determination of sugars and related substances. Analytical Chemistry. 28: 350-356.
11. Lowry, S., 1951. Determinants of extracellular protein secretion in Gram-negative bacteria, J. Bacteriol., 174: 3423-3430.
12. Wang, J.C., S.H. Hu, Z.C. Liang and C.J. Yeh, 2005. Optimtimization for the production of water soluble polysaccharide from *Pleurotus citrinopileatus* in submerged culture and its antitumor effect, Appl. Microbial. Biotechnol., 67: 759-766.
13. Bruhn, T.D., N.K. Jan and B. Laszlo, 1996. Antiviral and anticoagulant activity of polysaccharides from marine brown algae. Biochemistry Aspects Margarine Pharmocol., 14: 187-208.
14. Jian Fa Zhang, X.U. Xiang Hong, W.U. Hou Ming and L.I.U. Zhi Li, 2003. Structural Characterization of an Exopolysaccharide from the Myxobacterium *Sorangium cellulosum* NUST06. Chinese. Chemical. Lett., 14(1): 51-53.
15. Becker, A., F. Katzene, A. Puhler and L. Ielpi, 1998. Xanthangum biosynthesis and application: a biochemical genetic perspective. Appl. Microbial. Biotechnol., 50: 145-150.
16. Lee, J.H., S.M. Cho, K.S. Ko and I.D. Yoo, 1995. Effect of cultural conditions on polysaccharide production and its monosaccharide composition in *Phellinus linteus* (L13202). Korean J. Mycol., 23: 325-331.
17. Fang, Q.H. and J.J. Zhong, 2002. Effect of initial pH on production of *Ganoderma lucidium.* Process Biochemistry. 37: 769-776.
18. Burns, P.J., P. Yeo, T. Keshavarz, S. Roller and C.S. Evans, 1994. Physiological studies of exopolysaccharide production from the basidiomycetes *Pleurotus florida.* Enzyme Microb. Technol., 16: 566-572.
19. Costerton, J.W., Z. Lewaudowski, D.E. Caldwell, D.R. Korber and J.M. Lappin-Scott, 1999. Microbial biofilms. Annu. Rev. Microbiol., 49: 711-745.
20. Anselm Omoike and Jon Chorover. 2004. Spectroscopic study of extracellular polymeric substances from *Bacillus subtilis*: Aqueous chemistry and adsorption effects. Biomacromolecules. 5: 1219-1230.
21. Yuan, Z.L., 2003. *Lycium barbarum* polysaccharide production technique and determination of polysaccharide content along with molecular mass distribution, *Guangdong.* Chem. Ind., 3: 43-45.
22. Swardeker, J.S., J.H. Sloneker and A. R. Jeans, 1965. Quantitative determination of monosaccharides as their alditol acetates by gas-liquid chromatography. Anal Chem., 37: 1602-1604.
23. Hoebler, C., L.B. Jean, D. Agnes and D.L. Jean, 1989. Rapid acid hydrolysis of plant cell wall polysaccharides and simplified quantitative determination of their neutral monosaccharides by gas-liquid chromatography. J. Agric. Food Chem., 37(2): 360-367.
24. Dawson, R. and K. Mopper, 1978. A note on the losses of monosaccharides, amino sugars and amino acids from extracts during concentration procedures. Anal. Biochem. 84: 186-190.