Recent Research in Science and Technology 2010, 2(4): 01-05
ISSN: 2076-5061
www.recent-science.com

MICROBIOLOGY

SUCROSE AND MALTOSE AS CARBON SOURCES FOR FERMENTATIVE PRODUCTION OF POLYSACCHARIDE BY *ASPERGILLUS NIGER* VAN TIEGH.

N. Senthilkumar* and S. Murugesan

Division of Bioprospecting, Institute of Forest Genetics and Tree Breeding, Coimbatore -641 002, Tamilnadu, India

Abstract

Two carbon sources viz., sucrose and maltose were used to induce extracellular polysaccharide production in *Aspergillus niger*. Maximum production of polysaccharide 513 mg/100ml of culture filtrate was observed at pH 7, 10% sucrose concentration, 15 days of incubation. Polysaccharide production was increased with increase in growth of mycelium of A. niger in both the substrates. The optimum pH and incubation periods for the maximum production of polysaccharide were pH 7 and 15 days respectively. The polysaccharide produced by *A. niger* was the polyol of 3,4,6 tri-o-methyl D glucose with β-1-3 glycosidic linkage commonly known as glucan. The polysaccharide produced by this species may be composed of glucose and little amount of galactose and other oligosaccharides. Polarimetric studies revealed that the fractions containing glucose gave laevorotatory values of the optical rotation.

Keywords: *Aspergillus niger*, exopoplysaccharides, bioprospecting etc.

Introduction

One of the recent trends in microbiological investigations is the exploitation of some microorganisms to secrete exopolysaccharides (EPS) which are of commercial value as food additives (Gorin and Spencer, 1968), anti-tumor agents (Whistler *et al.*, 1976), flocculents (Zajic and Leduy, 1973), high-performance microbial gums (Lachke, 1996) and in paper & textile industries (Saxena and Kaushik, 1992). Extracellular polysaccharide production by a member of the Sclerotiniaceae was first described by Thomas (1930) who noted a gelatinous slime on the hyphal surface of *Sclerotina sclerotiorum*, which could be washed off and precipitated by alcohol. Willetts (1971, 1972) found copious production of extracellular mucilage by both *S. sclerotiorum* and *S. fructicola* but no characterization was attempted. Polysaccharide secreted by the later species was also the subject of a brief report by Feather and Malek (1972). Their results indicated that the material was highly branched glucan in which 1-3 linkages predominated. Pioneering attempt to isolate and characterize an extracellular branched D- Glucan from *Monilinia fructigena* was made by Archer *et al.*, 1977. Polysaccharide production by various bacteria is well known (Robyt *et al.*, 1974; Lopez and Monsan, 1980; Lee *et al.*, 1982). Ample investigations were carried out in *Pseudomonas* spp. (Williams and Wimpenny, 1980), *Rhizobium* sp. (Ghai *et al.*, 1981), *Xanthomonas* sp. (whitfield *et al.*, 1981), *Aeromonas* sp. (Ito *et al.*, 1982), *Hyphomicrobium* sp. (Kanamaru *et al.*, 1982) and *Aureobasidium* sp. (Hamada and Tsujisaka, 1983). However, there is paucity of information on the mycological production of extracellular polysaccharides. A few significant publications include those by Leal & Ruperez (1978) on *A. nidulans*, Leal-serreano *et al.*, (1980) on *Aureobasidium pullulans* and Ruperez *et al.*, (1983) on *Penicillium erythromellis*. Microbial polysaccharides have had attention because of their unique properties such as Antitumor agents - Curdlan, Blood expander - Dextran, pollution preventing agents - Xanthan and Food preservatives - Pullulan (Hamada and Tsujisak, 1983). Although polysaccharide produced by different species of *Aspergillus* is known, information on the production of EPS from *A. niger* and the nutritional and environmental factor affecting polysaccharide production have not been reported widely (Ruperez and Leal, 1979). The present study is an attempt to explore the possible potential of polysaccharide production and the composition of an extracellular polysaccharide isolated from the culture medium of *A. niger.*

Materials and methods

Isolation of microorganism

The fungal pathogen *A. niger* was isolated from the diseased larvae of *Eupterote geminate* Walker., a serious pest of verbinaceous tree species, *Gmelina arborea* (Roxb.) collected from Central Nursery, Institute of Forest genetics and Tree Breeding,

* Corresponding Author, *Email*: senthilnj@hotmail.com

Coimbatore, Tamilnadu, India. It was cultured on Sabourad agar medium and was incubated at 26-27º C for 6-8 days in the dark. Pure cultures of *A. niger* were maintained separately on slants of Sabourad agar medium supplemented with 1gm/li of yeast extract. The fungus was subjected to screening for polysaccharide production as per Leal and Ruperez (1978).

Screening for polysaccharide producing microorganism

The fungus was inoculated into a large test tube containing 15ml of the screening medium i.e., (gm/100ml) sucrose, 6; $NaNo_3$, 0.3; KCl, 0.05; Mg $So_4.7H_2O$, 0.005; KH_2PO_4, 0.1 and $FeSO_4$. $5H_2O$, 0.005 an initial pH 6 and incubated at 30°C as static liquid medium. Five days old cultures were heated at 80°C for 15min. Then two volumes of ethanol were added to the supernatant, and the resulting precipitate was collected by centrifugation (10,000-x g, 5 min). The precipitate was dissolved in water and the phenol H_2SO_4 method of Dubois *et al.*, (1956) used for quantitative estimation of the sugar contents.

Culture media and cultivation of *A. niger*

The EPS *A. niger* was grown in the 250 ml Erylinmeyer flask containing 100ml of the medium (g/100ml). One medium was sucrose and the other contained maltose with the following combinations (sugar 6, 8 and 10gm (sucrose & maltose); $NaNo_3$, 0.3; KCl, 0.05; Mg $So_4.7H_2O$, 0.005; KH_2PO_4, 0.1 and $FeSO_4$. $5H_2O$, 0.005. The media were adjusted to different pH 5,7 & 9 with 1N NaOH and different fermentation periods of 10 & 15 days. Each cotton-plugged flask was autoclaved at 120ºC for 15 min and then allowed cooling.

Innoculum

Fermentation medium was inoculated with ~6, 92, 000 spores/ ml from the stock culture and incubated at 30°C in static culture flask. The spore count was aided with the help of a Haemocytometer.

Isolation and purification of polysaccharides

The cultivated fungal cell mass was collected on a fine cheesecloth dried at 60°C overnight and weighed. The culture broth was heated for 15 min at 60°C and then cell free solution was obtained by centrifugation (8900xg, 15 min). The crude polysaccharide was separated from the supernatant by the addition of two volumes of ethanol and the precipitate that wound around the stirrer. The precipitation procedure was repeated thrice and the final product was dried at 60°C and ground to a fine powder. In an old culture the precipitate did not wound around the stirrer and had to be collected and washed by centrifugation (Leal & Ruperez, 1978).

Acid hydrolysis of polysaccharides

The polysaccharide was hydrolyzed with 2.5 N HCl at 105°C for 1, 2, 3 hrs in sealed evacuated tubes (Leal & Ruperez, 1978).

Analysis of polysaccharide

Chromatographic analysis of sugars

The sugars in the isolates were identified by ascending chromatography on Whattmann No 1 filter paper (0.22μ) using n-Butanol/pyridine/0.01N HCl (50:30:14, by volume) as solvent. The spots were developed with ammoniacal silver nitrate (Hough and Jone, 1962). For thinlayer chromatograms, Readymade TLC plate (Merck – 20x20cm, silica gel 60 F_{254}) was used was used as a support and butan1-01/ acetic acid/ water (3:1:1 by volume) as solvent (Shah and Loewv, 1967). Sugars on chromatogram were visualised by spraying silver nitrate in acetone followed by ethanolic sodium hydroxide (Trevelyan *et al.*, 1950).

Infrared spectrum

After running TLC, samples were scrapped off from the plates, pooled and eluted with distilled water and dried to get the compounds of interest in a solid from. Samples (2mg) were mixed with KBr and pressed into pellets of 13 mm size and infrared spectrum were recorded using Perkin - Elmer IR spectrophotometer (Model IR 577).

Mass spectrum

This valuable technique can be combined with other classical isolation methods help to identify the organic compounds unequivocally. In mass spectrometry, the sample was bombarded with an electron beam and converted into rapidly moving positive ions, which were then separated in the presence of a high magnetic field and characterised on the basis of change by mass ratio. The results were quantitatively recorded as a spectrum of positive ion fragments. The resulted mass peaks were identified by comparison with authentic reference data. The work was carried out in the Varian 4000 apparatus.

Chemical analysis of culture filtrate

The total carbohydrate was determined by anthrone method after the supernatant (Crude

polysaccharide solution) separated from culture broth. (Dreywood, 1946). The total protein present in the culture filtrate was estimated by using spectrophotometer as per the method of Lowry *et al.*, (1951). The nitrogen content was calculated from the protein values by dividing the factor 6.25 because 1mg of nitrogen is equal to 6.25 mg of protein. So this is protein nitrogen (Ghai *et al.*, 1981). Reducing sugar was estimated following dinitrosalicyclic acid method (Miller, 1972). Optical rotation of the polysaccharide fraction was determined with a Harnack HA.4001 microcell polrimeter. Total acidity was determined in the filtrate by titration with 0.1N NaOH to phenolphthalein end point (Leal - Serrano *et al.*, 1980).

Results and Discussion

Two carbon sources viz., sucrose and maltose were used to induce extracellular polysaccharide production in *Aspergillus niger* on 10 days and 15 days of incubation under three pH conditions (5,7, &9) and at three different substrate concentrations (Table 1&2). The maximum production of polysaccharides and mycelial growth was achieved within 15 days of incubation. Leal - Serrano *et al.*, (1980) reported that the maximum amount of polysaccharide was isolated from *Auerobasidium pullulans* after 15 days of incubation. This polysaccharide could be a cell wall component that the microorganisms continue to produce when there is very little demand for cell wall synthesis. It could also be cytoplasmic reserve that is produced in larger amounts than the fungus is able to store (Leal *et al.*, 1979). Whereas, maximum production of 86.9 mg polysaccharide /100ml was observed at pH7, 10% sucrose concentration, 15 days of incubation in *A. niger* by Ghareib in Egypt, 513 mg/100ml was recorded in the present study in exactly similar conditions of pH and sucrose concentration on 15 days of incubation and this disparity may be due to the difference in the strain of *A. niger* isolated from the insect.

Table 1: Effect of sucrose for the production of exopolysaccharide by *Aspergillus niger*

Substrate concentration	6%						8%						10%					
PH	5		7		9		5		7		9		5		7		9	
Incubation period (dys)	10	15	10	15	10	15	10	15	10	15	10	15	10	15	10	15	10	15
Vol.of the filtrate (ml)	80	75	75	65	70	75	75	60	65	55	70	55	65	55	60	50	65	60
Wt.of the Polysaccharide*	0.01	0.04	0.02	0.19	0.02	0.10	0.08	0.18	0.35	0.30	0.17	0.16	0.32	0.36	0.44	0.51	0.24	0.48
Wt.of the mycelium*	0.51	1.37	0.83	1.89	0.79	1.61	2.39	2.13	2.84	2.57	2.57	2.48	2.26	2.07	2.72	2.36	2.07	2.21
Carbohydrate*	2.83	0.02	2.44	5.48	3.44	0.92	3.75	0.89	9.17	9.71	7.44	2.98	5.89	2.61	6.27	9.71	3.99	4.39
Reducing sugar*	0.31	0.96	1.36	1.48	0.75	0.75	0.45	1.13	0.50	1.30	0.36	0.31	1.26	0.65	1.48	1.36	1.25	1.08
Protein*	2.32	1.43	0.19	0.17	0.30	0.75	1.06	1.97	0.01	0.89	0.84	1.03	2.09	2.17	1.75	1.47	1.91	1.91
Nitrogen*	0.37	0.23	0.03	0.11	0.05	0.12	0.17	0.32	0.16	0.14	0.14	0.17	0.34	0.35	0.28	0.24	0.31	0.30
Total acidity	0.45	0.71	0.33	0.3	0.33	0.37	0.56	1.08	0.37	0.67	0.45	0.6	0.56	0.93	0.52	0.75	0.45	0.67

* gm/100ml

Table 2: Effect of maltose for the production of exopolysaccharide by Aspergillus niger

Substrate concentration	6%						8%						10%					
PH	5		7		9		5		7		9		5		7		9	
Incubation period (days)	10	15	10	15	10	15	10	15	10	15	10	15	10	15	10	15	10	15
Vol.of the filtrate (ml)	70	70	70	70	70	80	80	75	60	60	80	65	65	80	60	60	70	70
Wt.of the Polysaccharide*	0.04	0.17	0.13	0.21	0.11	0.11	0.13	0.28	0.32	0.48	0.20	0.36	0.11	0.17	0.35	0.47	0.11	0.40
Wt.of the mycelium*	0.84	1.41	0.91	0.85	0.17	0.27	0.22	1.18	0.84	1.39	0.62	1.18	0.21	1.12	0.81	1.66	0.44	1.16
Carbohydrate*	6.25	0.05	7.43	1.98	6.27	0.71	0.21	0.43	9.71	0.65	6.27	0.09	0.13	0.37	2.27	3.54	0.45	1.22
Reducing sugar*	0.22	0.13	1.02	0.31	0.61	0.22	0.26	0.13	0.96	0.22	0.60	0.17	0.17	0.08	0.08	0.70	0.45	0.13
Protein*	1.79	0.41	0.17	1.01	1.0	0.18	2.82	2.60	0.73	0.32	1.51	0.39	0.37	2.70	0.18	0.19	0.24	0.66
Nitrogen*	0.29	0.07	0.03	0.02	0.16	0.03	0.45	0.42	0.12	0.05	0.24	0.06	0.06	0.43	0.03	0.03	0.04	0.11
Total acidity	0.52	0.45	0.3	0.26	0.22	0.41	0.93	1.16	0.62	0.33	0.6	0.12	1.12	0.37	0.71	0.3	0.82	0.18

* gm/100ml

Previous workers have studied the utilization of several carbon sources Viz., glucose and sucrose in 13 species of *Aspergillus* (Leal & Ruperez, 1978), as well as nitrogen sources viz., L-alanine, L-aspargine, L-aspartic acid, L-gultamine, L-gulutamic acid, L-leucine, L-phenylalanine, L-valne, potassium nitrate and diammonium tartarate in *A. nidulans* (Ruperez and Leal, 1979). Investigations were also carried out to induce extracellular polysaccharide production by utilizing both carbon (glucose) and nitrogen (Casiamino acids) and

L-asparagine) in *A. parasiticus* (Ruperez and Leal, 1981) and *A. alliaceus* (Gomez-Miranda and Leal, 1981). Leal-Serrano *et al.*, (1980) opined that among the carbon sources investigated for polysaccharides production, maltose (68.5 mg/100ml) and mannose (66.5mg/100ml) proved to be the best whereas manitol 12.4mg/100ml) gave the lowest polysaccharide yield. Interestingly in the present study, 6-10% sucrose as the carbon source yielded fairly high level of extracellular polysaccharide 192-513 mg/100ml in pH7 on 15 days of incubation in *A. niger* (Table 1).

Chromatographic analysis of sugar composition

Acid hydrolysis of both the fractions, one pertaining to sucrose and the other to maltose followed by thinlayer chromatography revealed glucose as the predominant monosaccharide which is in conformity with the observations of Archer *et al* (1977) on *Monilinia fructigena*. It was also found that, HPLC analysis indicated the presence of galactose.

Polarimetric studies of the fractions containing glucose gave the laevorotatory values of the optical rotation as pointed out by Ruperez *et al.*, (1984) on *Penicillin allababadens* and the configuration of polysaccharide fraction could be ascertained with the help of IR spectroscopy. It revealed that the polyol have β-1-3 glycosidic linkage. This polyol may be of 3,4,6 tri-o-methyl D glucose. It was confirmed by mass spectrum. The common name is glucan. Interestingly Leal and Ruperez (1978) observed the same result on *A. nidulans*.

Chemical composition of culture filtrate

Total carbohydrate, reducing sugar, total protein, nitrogen (calculated from protein values), and total acidity of the culture filtrates at the end of the aforementioned experiments were estimated and the results are presented in Table 1&2. Higher values of carbohydrate (0.65gm/100ml to 9.17gm/100ml and reducing sugar (0.2 to 1.48gm/100ml) were recorded in all the three different substrate levels at pH7 where as protein and nitrogen showed a decline at that point. Leal and Ruperez (1978) observed similar carbohydrate and protein values in pH 6.5 in *A. nidulans*. Total acidity was maximum at pH 5 and minimum at pH7.

Acknowledgement

Authors are grateful to the Director, IFGTB for providing facilities during the course of this study. Dr. K.P. Sanjayan, G.S. Gill Research Institute, Guru Nanak College, Chennai is greatfully acknowledged for his critical comments on the manuscript.

References

Archer, S.A., Clamp, J.A. and Daniel Miglione. (1977). Isolation and partial characterisation of an Extracellular branched D-Glugan from Monilinia fructigenia Journal of General Microbiology, 102: 157-167.

Drywood, R. (1946). Quantitative test for carbohydrate material. Industrial and Engineering Chemistry. Analytical Edition, 8, 499.

Dubios, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A ., and Smith, F. (1956). Anal. Chem., 28: 350

Feather, M. S., and Malek, A. (1972). A highly branched exocellular D-Glucan from Monilinia fructicola. Biochemica et Biophysica acta, 264, 103-105.

Gorin, P. A. J., and Spencer, J. F. T. (1968). Structural chemistry of fungal polysaccharides. Advances in carbohydrate chemistry, 22, 367 - 417.

Gomez-Mirinda and Leal, J. A. (1981). Extracellular and cell wall polysaccharides of Aspergillus allaiaceus. Trans. Br. Mycol. Soc., 76 (2), 249-253.

Ghai, S. K., Makoro Hismatsu, Akinori Amemura, and Tokuya Haroda. (1981). Production and chemical composition of extracellular polysaccharide of Rhizobium. Journal of General Microbiology, 122, 3-40.

Hough, L. A., and Jones, J. K. W. (1962). Chroamatography on paper. Methods in carbohydrate chemistry,1, 21-31.

Hamada, N., and Tsusisaka, Y. (1983). The structure of carbohydrate moiety of an acidic polysaccharide produced by Auerobasidium sp. Kl. Agric. Biol. Chem., 47 (6), 1167-1172.

Ito, K., Yoshino, H., and Veda, S. (1982). Structure of acidic polysaccharide produced by strain no.626 of Aeromonas hydrophila. Agric. Biol. Chem., 46 (8): 2051-2056.

Kanamaru, K., Hieda, T., Iwanura, Y., Mikami, Y., and Kisaki, T. (1982). Isolation and characterisation of Hypomicrobium. Sp. and polysaccharide formation from methanol. Agric. Biol. CheM., 46 (10): 2411-1417.

Lachke, A. 1996. Sticky bugs. Science Reporter, 28, 28-29.

Leal, J. A., and Ruperez, P. (1978). Extracellular polysachharide production by Aspergillus nidulans. Trans. Br. Mycol. Soc., 70. 115-120

Leal, J.A., Ruperez, P., and Gomez-Mirinda, B. (1979). Extracellular B-Glucan Production by Botytis cinerea . Trans. Br. Mycol. Soc., 72 (1): 172-176

Leal-Serrano, G., Ruperez P., and Leal, J.A. (1980). Acidic polysaccharide from Auerobasidium pullulans. Trans. Br. Mycol. Soc., 75, 57-62.

Lee, H. C., Lee, M. G., Kao, Y. S., and Lee, S. C. (1982). Nat. Sci. Coun. Mon. 10, 651.

Lopez, A., and Monsan, P. 1980. Biochemie, 62, 323.

Lowry, O. H., Rosebrough, N. J., Farr, A.L., and Randall, R. J. (1951). Protein measurement with the Folin Phenol reagent. Journal of Biological Chemistry, 93, 265-275.

Miller, G. C. (1972). Annual. Chem: 31: 426.

Ruperez, P., and Leal, J. A. (1981). Extracellular Galactosaminogalactan from Aspergilus parasiticus. Trans. Br. Mycol. Soc., 77 (3), 621-625.

Ruperez, P., and Leal, J. A. (1979). Utilisation of nitrogen sources by Aspergillus nidulans for extracellular polysaccharide production . Trans. Br. Mycol. Soc., 72 (2), 291-297.

Ruperez, P., Gomez-Mirinda, B., and Leal, J. A. (1983). Extracelular B-Glugan from Pencillium erythromellis. Trans. Br. Mycol. Soc., 80. (2) 313-318.

Ruperez, P., Gomez-Mirinda, B., and Leal, J. A. (1984). Acidic polysaccharide from Pencillium allahabadens. Can. J. Microbiol., 30: 1157-162.

Robyt, J. E., Kimble, B. K., and Walseth, T. F. (1974). Arch. Biochem. Biophys., 165.

Saxena, S., and Kaushik, B. D. (1992). Polysaccharide (Biopolymers) from Halotolerant cyanobacteria. Indian Journal of Experimental biology, 30. 433-434.

Shah, R. H., and loewvs, F. (1967). Synthesis of methyl D-galacturonate from D. Galactose or D-galacturonic using methyl -C14 iodide. Carbohydrate.

Thomas, R. C. (1930). Composition of fungal hypae II sclerotinia. *American Journal of Botany.* **17**, 71-778.

Trevelyan, W. E., Procter, D. P., and Harrison, J. S. (1950). Detection of sugars on paper chromatograms. Nature, London 166: 444

Whistler, R. L., Bushway, A. A., and Singh, P. P. (1976). Non cytotoxic, antitumor polysaccharides. Advances in carbohydrate chemistry and Biochemistry, 32, 235-275.

Whitfield, C., Sutherland, I. W., and Cripps, R. C. (1981). Surface polysaccharide in mutants of Xanthomonas camestris. Journal of General Microbiology, 124, 385-392.

Willets, H. J. (1971). The survival of fungal sclerotia under adverse environmental conditons. Biological Reviews, 47, 515-536.

Willets, H. J., (1972). The morphogenesis and possible evolutionary origins of fungal sclerotia. Biological Reviews, 49.

Williams, A. G., and Wimpenny, J. W. T. (1980). Extracellular polysaccharide Biosynthesis by Pseudomonas NCIB 11264. Studies on precursor - Forming Enzymes and factors affecting Exopolysaccharide production by washed suspension. Journal of General Microbiology, 116, 133-141.

Zajic, J. E., and Le Duy, A. (1973). Flocculant and chemical properties of a pullulan from Pullularia pullulans Applied Microbiology, 25. 628-635.