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Production of Exopolysaccharide from Local Fungal Isolate

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Abstract

The study investigated in isolation of 26 fungal isolates belonging to 6 different genera viz., *Penicillium* sp., *Aspergillus* sp., *Fusarium* sp.,*Alternaria* sp., *Rhizopus* sp. and *Phoma* sp. Were screened for exopolysaccharide production. Glucose in culture media was studied to select the medium that gives a maximum production of exopolysaccharide by *Penicillium* sp., Exopolysaccharide was isolated by ethanol precipitation. The medium which contains glucose had been selected to get the heights production of exopolysaccharide. Fermentation conditions were further investigated to optimize exopolysaccharide production by *Penicillium* sp., The optimum substitution ratio, temperatures, pH and incubation periods for the maximum production of the polysaccharide were 100% , 30 ˚C , pH 5 and 9 days respectively. Characteristic of exopolysaccharide compounds were observed in the FTIR spectrum. Thin layer chromatography of the hydrolyzed polysaccharide showed that the exopolysaccharide production was heteropolysaccharide consists of galactose, glucose and mannose.

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Keywords

Filamentous fungi;
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Introduction

In the last few years, much more interest there has been a growing in extracellular polysaccharides production, especially from microorganisms, it has attracted the interest of the many researchers attributed to their industrial importance in varied food and bio-applications, Microbial exo-polysaccharides (EPSs) generally consist of monosaccharides units joined by glycosidic bonds and some non-carbohydrate substituents and its was produced by both prokaryotes and eukaryotes which are rich sources of enzymes, are also being intensively investigated as a permanent source of polysaccharides for industrial applications[1]. Xanthan was produced from *Xanthomonas*[2] Gellan from *Sphingomonas paucimobilis* Also produced from fungi such as *Auerobasidium pullulans*[4] *Saccharomyces cerevisiae* [5]*Penicillium* [6]*Fusarium* [7]*Aspergillus*[8,9]. The fungi are often dispersed in the media of growth while some are linked to the cell wall which are either part of fungal cell wall or excreted extracellularly to play roles in cell protection or

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attachment to surfaces[10]. Exopolysaccharides have been isolated from fungi as an active secondary metabolite and occurs as a fixation system to the substrate, and some of them may have biological activities or pathogenic effects over the host[11]. The fungi of Ascomycota and Basidiomycota produced a maximal yield of Exopolysaccharides reached to 42.24 g l^{-1}, This depended mainly on the culture conditions and type of strain[12].These EPSs have been used for biomedical applications and it is widely used as an additive in the food industry, and as a rheology modifier: it is used for thickening, stabilizing, emulsifiers, gelling agent, bioadhesives and antioxidants[13,14].

The present study aimed to produce the Exopolysaccharides from local fungi isolate to be used in the many foods and pharmaceutical applications.

Material and Methods

Monosaccharides was included :d-glucose, d-mannose(Merck), maltose (Scharlau),and d-galactose (Sigma–Aldrich). Dextran standards were gotten from BDH, Potato Dextrose Agar (Himedia).

Isolation

Fungi were isolated from soil, which was collected from different parts of Basrah city, such as paper factory , Agriculture College and Shatt Al Arab River. Soil was taken from a depth range of 15-20 cm. A total of one gram of soil aseptically suspended in sterile peptone water (0.1%) and serial dilutions were made to 10^{-6}, Transfer 1 ml of suspension of each dilutions to Petri dishes containing of Potato Dextrose Agar media. Chloramphenicol was added to the medium, then incubated at 28± 2 ^{0}C for 5-7 days. Colonial morphology and microscopic examinations of the various isolates of pure cultures were used to diagnose and identify Depending on the reference[15,16]. The percentage of frequency of each isolate was calculated according to the following equation:

$$M\% = (N \times 100)/T$$

Where M is the coded value of the percentage of mean frequency, N is the Number of a single mold isolates,T is the Total number of isolates in the region.

Preparation of Inoculum

The spores from a fully sporulated slant were dispersed in 10 ml of 0.1% Tween 80 by gently scrapped them with a sterile loop .The number of spores was counted using a hemocytometer, diluted to 31×10^7 spores/ml[17].

Screening, Isolation and Purification of Exopolysaccharide Producing from Fungi Isolate

The isolates were screened for best isolate produced exopolysaccharide according to[9],The fungus were inoculated in a conical flask (250 ml) with 50 ml of the medium containing: sucrose, 6; $NaNO_3$, 0.3; KCl, 0.05; $MgSO_4.7H_2O$, 0.005; KH_2PO_4, 0.1; $FeSO_4.5H_2O$, 0.005 g/100 ml at pH 6 and incubated at 30 °C for 7 days. After that cultures was heated at 80 °C for 15 min. Then filtered through cheesecloth and supernatant mixed with two volumes of ethanol (95%) and stored overnight at 5 ^{0}C and precipitate was recovered by centrifugation at 5000 xg for 10 min, EPS was washed 3 times with 10% Trichloroacetic acid (then removed by repeated washing with acetone). the EPS was dried at 60 ^{0}C and the total sugars were determined as glucose equivalents according to the method of[18,19].

Biomass Estimated

The biomass was estimated according to[20] by filtration of cells after fermentation and drying at 50 °C until weight constant and expressed in g/l00 ml.

Effect of Culture Parameters on Exopolysaccharide Production

The optimum medium composition for enhancing exoploysaccharide production was determined by studying the effect of the carbon sources, sucrose was substituted by either glucose and lactose, all at a concentration of 6 g/l00ml, Moreover ,The different temperatures (25, 30 and 35 ^{0}C), pH (4, 5,6 and 7) and incubation period (3,4,5,6,7,8,9 and 10 days) for EPS production by *Penicillium* sp. was studied by using a conical flask containing 100 ml of liquid medium.

Fourier-Transform-Infrared Spectroscopy (Ftir)
Exopolysaccharide was pressed into a 1 mm pellets with KBr powder, and then measured on an FTIR spectrometer (Jasco, Japan) in the wave number of 4000–400 cm^{-1}.

Thin-Layer Chromatography of Sugar Components
Polysaccharide was hydrolysed with HCl in sealed tubes in an oven at 105 ^{0}C. The sugars were identified in the hydrolysates were applied to silica gel 60 TLC plate. The solvent system for sugars was 1-butanol:piridine: water (6:4:3 v/v/v) and the spots were developed with 10 mL of 95% of sulphuric acid in 82 ml of methanol[21].

Statistical Analysis
The Statistical Analysis System - SAS- was used to analyse the results[22]. The results compared statistically to standard deviation(±SD) for three replicates per treatment.

Results and Discussion
Table 1 shows that 6 different fungal isolates were diagnosed such as *Penicillium* sp., *Aspergillus* sp*., Fusarium* sp*., Alternaria* sp*., Rhizopus* sp.and *Phoma* sp. The study showed that the genus of *Penicillium* and *Aspergillus* were found to occur frequently in all the regions (Paper Shatt. Agri. Soil) and thus have the maximum variety of occurrence (30.76 and 34.61% respectively). As these two genera were isolated from about 65% of the soil samples. This was consistent with what many articles to the prevalence and spread of these two genera in nature. Whereas the lowest occurrence frequency was shown by *Alternaria, Rhizopus* and *Phoma*[1,8].

Table 1: Percentage of occurrence frequency of various fungi genera isolated from soils

No	Fungal isolates	Soil from			Mean of frequency %
		Paper	Shatt.	Agri.	
1	*Penicillium*	3	2	3	30.76
2	*Aspergillus*	2	3	4	34.61
3	*Fusarium*	-	1	3	15.38
4	*Alternaria*	-	-	2	7.69
5	*Rhizopus*	1	-	1	7.69
6	*Phoma*	-	-	1	3.84

The fungal isolates were screened for producing exopolysaccharide when grown in a sucrose medium. Among these were shown in Table.2, *Penicillium* sp. A2 produced the maximum amount of exopolysaccharides. The yield of crude exopolysaccharide and mycelial growth from the fermented broth were reached 0.25 and 1.82 g/100 ml respectively. Hence this isolate was selected for further studies. The study detect no similarity in the variety and the distribution pattern of these EPS producing fungi in the region. This may be due to the differences in the moisture levels, temperatures, type of available substrates their pH and nature of the soil and normal of regions[20]. All these factors affect on the characteristics and quality of the EPS, This result agreed with the many studies which have utilization of different fungi for exopolysaccharid production viz., *Penicillium, Fusarium, Aspergillus, Alternaria* [23,6,7,9]. Fungi produce a large diversity of polysaccharides which can be isolated from the cell wall, cytoplasm, or culture medium and its continue to produce EPS when there is a little demand for cell wall synthesis[19].

Table 2: Screening the polysaccharides producing fungi (g/100ml) on sucrose medium

No.	Genera	Biomass g/100 ml	Exopolysaccharide g/100 ml
1	*Penicillium* A1	1.32±0.033	0.055±0.001
2	*Penicillium* A2	1.82±0.048	0.25±0.013
3	*Penicillium* A3	1.22±0.029	0.029±0.001
4	*Penicillium* A4	0.96±0.012	0.014±0.001
5	*Penicillium* A5	0.92±0.015	0.022±0.001
6	*Penicillium* A6	0.32±0.021	0.099±0.001
7	*Penicillium* A7	0.86±0.014	0.081±0.002
8	*Penicillium* A8	0.74±0.012	0.054±0.001
9	*Aspergillus* A9	1.78±0.044	0.15±0.023
10	*Aspergillus* A10	1.23±0.025	0.05±0.001
11	*Aspergillus* A11	1.26±0.026	0.012±0.001
12	*Aspergillus* A12	1.96±0.034	0.17±0.021
13	*Aspergillus* A13	0.93±0.031	0.06±0.002
14	*Aspergillus* A14	0.62±0.012	0.03±0.001
15	*Aspergillus* A15	0.33±0.007	0.06±0.003
16	*Aspergillus* A16	0.74±0.011	0.02±0.001
17	*Aspergillus* A17	0.59±0.021	0.03±0.002
18	*Fusarium* A18	0.73±0.016	0.06±0.003
19	*Fusarium* A19	0.89±0.013	0.01±0.001
20	*Fusarium* A20	0.92±0.018	0.02±0.001
21	*Fusarium* A21	1.48±0.020	0.21±0.051
22	*Alternaria* A22	1.48±0.015	0.13±0.032
23	*Alternaria* A23	0.35±0.010	0.08±0.009
24	*Rhizopus* A24	1.25±0.011	0.04±0.003
25	*Rhizopus* A25	0.82±0.004	0.01±0.001
26	*Phoma* A26	0.94±0.006	0.08±0.005

The optimum conditions for exopolysaccharide production were studied and The results obtained in the Fig.1a which shows that the exopolysaccharides yield from the fermented broth media contains glucose was reached 0.284 g/100 ml and mycelial growth 2.15 g/100ml. when *Penicillium* sp. was grown in sucrose medium and lactose EPS production was much lower than monosacchride containing medium (0.258 and 0.191 mg/100 ml respectively). Thus, glucose was used as a carbon source and The substitution ratio of sucrose with glucose was also investigated. Fig.1b shows that 100% was the best and used in further studies. Previous studies have indicated that the concentration of carbon source was one of the most important factors in reaching to high-level exopolysaccharide production[24], Glucose is the main carbon source of many fungi[12,25] was used glucose as a carbon source of *Aspergillus* sp. in the medium gave the highest yield of EPS. Also [26]obtained high EPS when used glucose as a carbon source of *Penicillium vermiculatum*. The effect of carbon source on EPS and mycelial growth production using isolating was reported to be dependent on the isolate, sugar type and the cell carbohydrate metabolism properties[1].

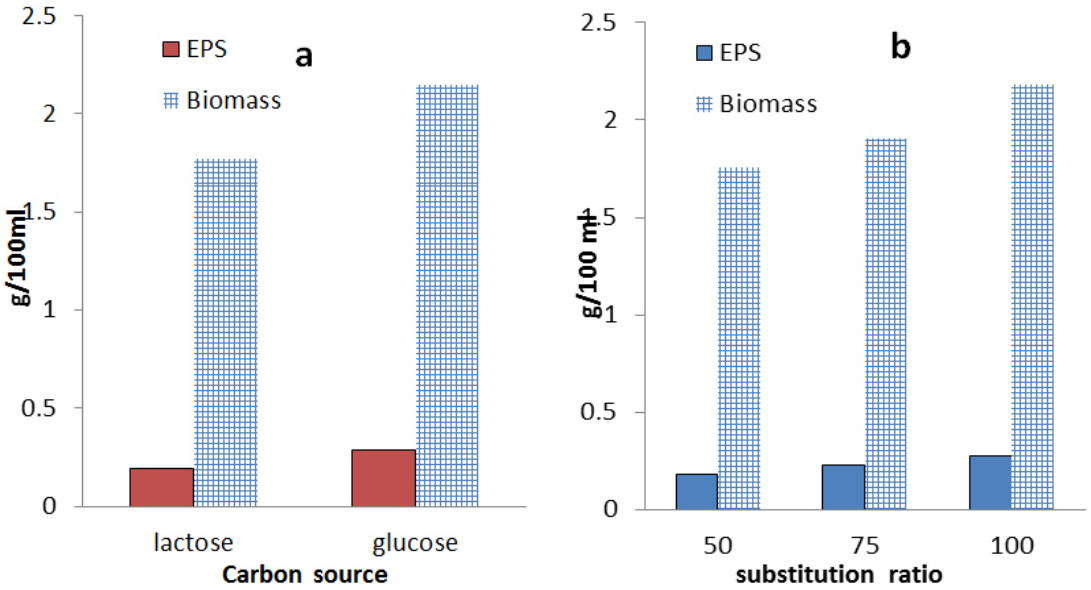

Fig.1: Effect of (a) carbon source (b) substitution ratio on Exopolycaccharide (EPS) Production by *Penicillium* sp.

The effect of incubation time on the polysaccharide composition was tested as illustrated in Fig. 2a. Cultures were harvested between 3 to 10 days after inoculation and the polysaccharide concentrations were the highest at 9 days and give a corresponding maximum value 0.314 g/100 ml. After the optimum incubation time there was a decrease in the quantity of EPS in the culture. Long incubation period contributed to decrease of polysaccharide concentrations in the broth medium and was related to the formation of low molecular EPS, which may well be due to enzymatic degradation, as has also been reported with other EPS. The optimum incubation time for exopolysaccharide synthesis in cultures of the fungi strains generally ranges between 3 - 40 days[12]. That's results agree with[23] who found that 9 days incubations is the best for exopolysaccharide production with *Alternaria alternate*. While[7] obtained the highest of exopolysaccharide after 13 days of incubation from *Fusarium* sp., Another reflective factor that affected on exopolysaccharide produced from fungi was pH of culture medium.The results in Fig.2b Showed that the optimal pH for exopolysaccharide was6. However, at pH levels higher or lower than 6, biomass and EPS concentrations were decreased. This may be due to changes in H^+ ion concentration in the medium, the solubility of salts, affect cell membrane function, the ionic state of substrates, cell morphology, the uptake of various nutrients, and product biosynthesis. Interestingly [26]observed similar EPS value in pH 6.3 in *Penicillium vermiculatum*, Others find optimum pH for exopolysaccharid from fungi was favored low pH with a range between pH 3.0 to 6.5[1].

The results of optimal temprature for exopoly-saccharide production by *Penicillium* sp. shown in Fig.3 , the optimum temperature was found at 30 ˚C its reached to 0.319 g/100 ml while the isolate produced was low exopolysaccharide concentration at 25 and 35 °C.

Similar results were obtained by[23] when found that the temperature at 30 °C is the best for exopolysaccharide production with *Alternaria alternate*. Also[20] obtained 4.60, 3.86 g/L of EPS at 30 C of *Aureobasidium* sp. and *Penicillium* sp. respectively. The fungi of Ascomycota and Basidiomycota strains produced a maximum yields of exopolysaccharide at a temperature range between 20 to 30 °C[12] .

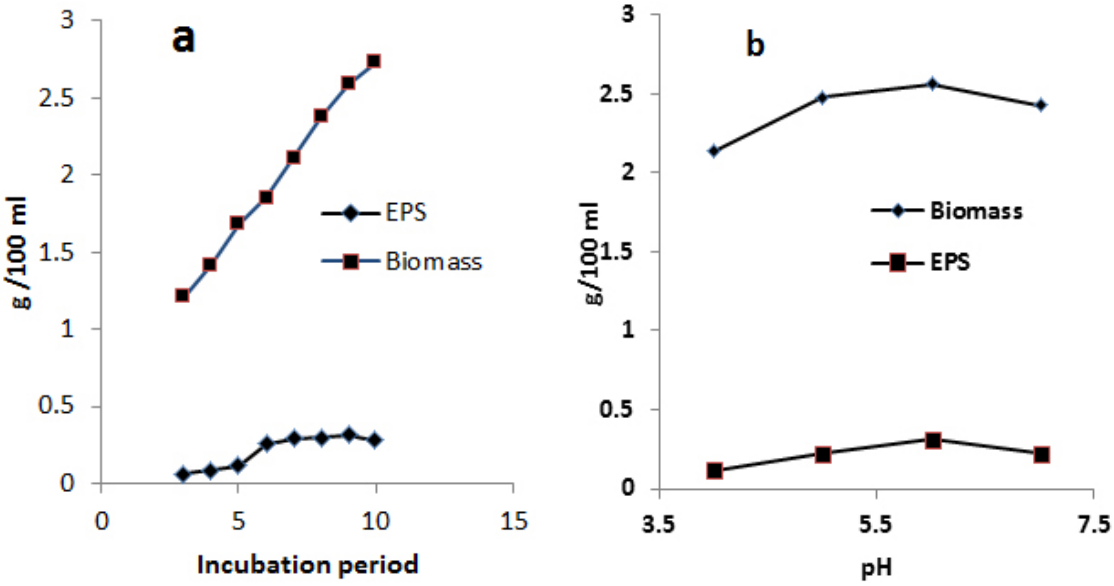

Fig.2: The effect of different incubation periods (a) pH value (b) on Exopolycaccharide (EPS) Production by *Penicillium* sp.

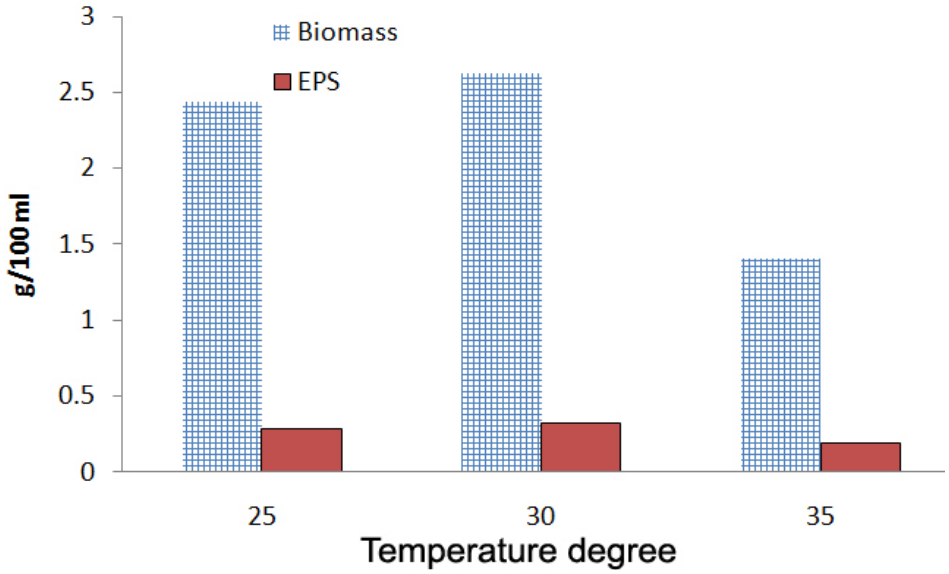

Fig. 3: The effect of temprature in Exopolycaccharide (EPS) Production by *Penicillium* sp.

FTIR Analysis

FTIR is an effective technique that works on the principle that group of bonds vibrates at characteristic frequencies. It can be employed to detect functional groups, and for characterizing covalent bonding. The infrared spectrum of exopolysaccharide is shown in Fig. 4. The signals at 3410 and 2935 cm^{-1} were attributed to the stretch vibration of O -H and C- H bond, respectively. bands at 1620 cm^{-1} was attributed to the vibrations of HOH . The signal at 1057 cm^{-1} was assigned to the stretch vibration of C-O and change the angle, vibration of O- H,

The bands at 1324 $cm^{-1}$was the absorption peaks of variable angle, vibration of C- H bond and The region of 1000–1200 cm^{-1} has showed an intense band which is characteristic of stretches C-O-C and C-O of the alcohol groups in carbohydrates.[27,28]. The band at 1030 cm^{-1} is characteristic of polysaccharide compounds[29]. These bands indicate that the substance is a polysaccharide[14].

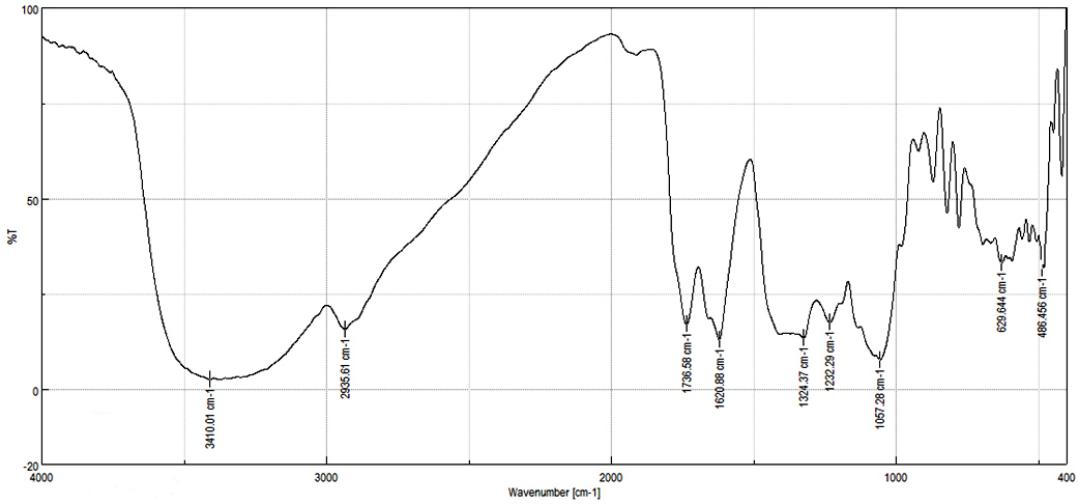

Fig.4: FT-IRspectrum of Exopolycaccharide from *Penicillium* sp.

Identification of Exopolysaccharide by TLC

The purified exopolysaccharide was analyzed by thin-layer chromatography after acid hydrolyzate and compared with glucose, galactose, manose and maltose to detect sugar monomers present in EPS. Table 3 show 3 sugars with Rf values identical to those of genuine glucose, galactose and mannose were detected with systems of solvent. 1-butanol:piridine: water, the Rf values for S1, S2, and S3 were 0.45, 0.48 and 0.52 respectively.

Table 3: Identification of sugar components of polysaccharide

Sugar type	Rf values
Glucose	0.49
Galactose	0.44
Mannose	0.54
Maltose	0.59
S1	0.45
S2	0.48
S3	0.52

Hence, the investigation isolate showed a similarity with the above in respect of the composition of EPS. [8]reported the presence of glucose, galactose and other oligosaccharides in EPS secreted by *Aspergillus niger* van tiegh, Also [6]found 3 sugars included mannose, glucose and galactose, whereas their glucuronic acid contents.

Conclusions

In conclusion, This study was enhanced the culture media, which developed for the production of exopolysaccharide, also, showed that *Penicillium* sp. could use as an existing carbon in glucose for exopolysacchride production. polysaccharides isolated from fungus *Penicillium* sp. was mainly consisted of glucose, mannose and galactose. Overall, this study can open a door for more studies so as to achieve even larger production of EPS from this isolate and additionally to clarify their actual composition and structures and their biological activities.

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