Plant Archives Vol. 18 No. 1, 2018 pp. 806-810 ***ISSN 0972-5210***

ISOLATION AND SCREENING OF FUNGAL ISOLATES : ABILITY TO POLLUTANT PRODUCTION AND DETERMINATION OF OPTIMUM CONDITION

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Abstract

Fifty fungal strains were isolated from holy *Karbala* governorate , taken from (Apple rotten. Olive tree leaves. River water, Soil Agricultural land) samples. The fungi were tested for their ability to to the exopolysaccharide (pullulan) production used as the standard media the sucrose is unique carbon source. The strain of *Aureobasidium pullulans* 1N and *Aureobasidium pullulans* 2N*, Rhizopus* spp 2N produced pullulan.

The optimum cultural and ecological condition for pullulans Production from *Rhizopus* spp (chosen because he had best of production). These condition were represented by the type and the concentration of carbon and nitrogen. Results revealed that , using production medium containing Date syrup as a source for carbon and ammonium chlorate as a source for nitrogen, as atotal concentration for mineral salts source . The medium was inoculated by using shaking inocular with 28 ÚC at 150 rpm /min for 72 hrs. The chemical structure of crude pullulans was produced from *Rhizopus* spp 2N identified by using Fourier transform-infrared (FT-IR) spectroscopy .

Key words : Biopolymers, *Rhizopus* spp, Pullulan, *Aureobasidium pullulans*, FT-IR, Exopolysaccharide.

Introduction

PULULLAN is a biopolymer with unique properties. It is a polysac-charide produced extracellularly by the fermentation of carbohydrate (starch/sug-ar) by the non-pathogenic and non-toxigenic strain of *Aureobasidium pullulans* (Mishra and Vuppu, 2012; Gaur *et al*., 2010). It has been found in soil, in lake water, on leaves, on weathered wood (Mirzwa-Mróz *et al*., 2014). *Aureobasidium pullulans* is a yeast-like Ascomycete (Order Dothideales, Family Dothideaceae). This fungus is polymorphic in its life cycle, exhibiting hyphae, conidiospores, blastospores and chlamydospores (Punnapayak *et al*., 2003).

Chemically pullulan properties, it consists of repeated unit of maltotriose units where three glucose units in maltotriose are connected by an alpha-1,4 glycosidic bond and consecutive maltotriose units are connected to each other by an alpha-1,6 glycosidic bond. This typical linkage pattern in fact render some distinctive physical traits which are different from many known polysaccharide (Gaur *et al*., 2010). The chemical formula of pullulan is $C_6H_{10}O_5.H_2O$ and the molecular weight varies from about 10 to 3 000 kDa. The essentially physical and biological properties is a water-soluble, white powder, odorless, flavorless, stable, non toxic, non immunogenic, biodegradable and bioadhesive (Hayashibara, 2017; Ferreira *et al*., 2015; Ganduri *et al*., 2016. Widespread production of Pullulan has far been limited due to high production costs. For Pullulan production to become more economically feasible, better fungal strains as well as cheaper feedstock and purification methods are needed. Genetically modified fungi will allow the use of cheap and abundant sources, such as household waste, agricultural and industrial waste, waste water, etc. for producing large amounts of Pullulan. Cheap, safe and efficient purification methods can then be used to recover Pullulan with high purity (Stankovic, 2005; Oliveiraa *et al*., 2015). This polysaccharide is of economic importance with increased application in food, pharmaceutical, medical applications, agricultural , chemical industries and can be used as potent antimicrobial agent for various biomedical applications (Ferreira *et al*., 2015; Gandurfi *et al*., 2016). The major step in the process and production of pullulan is the proper cost-effective downstream processing for its use in the various pharmaceutical formulations (Mishra adn Vuppu, 2013; Combie, 2006).

Materials and Methods

Fungal isolation

Fungal samples were collected from (Apple rotten, Olive tree leaves, River water , Soil Agricultural land) samples at holy *Karbala* governorate were studied. All fungal colonies were selected and subcultured on potato dextrose agar (PDA) (Himedia). Fungal isolates was diagnosed by adopting phenotypic and microscopic examination according to Mirzwa-Mróz *et al.* (2014).

Inoculum preparation

Fungal isolates were activated through growth in PDA agar slant at 28ºC for 48 h, then were transferred to 500 mL flask containing 100 mL of the standard media this used in pullulan production. The flask was incubated at 28ºC in a rotary shaker at 150 rpm. This seed culture was used to inoculate the production medium (Singh *et al*., 2012).

Production and Extraction of pullulan

The fungal strains used in this study was screening Efficiency for produce pullulan by growth in the standard media contained: 30 g of sucrose, 5.0 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$ and 1.0 g NaCl 1.0 per liter of distilled water. The pH value was adjusted to 6.0 with 1 M NaOH and, subsequently, the medium was sterilized at 121°C for 15 minutes. The carbon source concentration added to this medium after autoclaving .Each flask was shaken (150 rpm) for a period of 3 days at 28ºC (Oliveiraa *et al*., 2015; Mishra *et al*., 2011; Singh, 2015)

Preciptation of pullulan

After incubation time in standard media broth at 28°C, the culture medium was heated at 100ºC in water bath for 15 minutes, cooled to room temperature and centrifuged at 6,000 rpm for 20 minutes to remove fungal cells and other precipitates. Ten milliliters of the supernatant were transferred into other flask (50 ml) after filtered using whatman filter paper No. 1 and added 20 ml of cold ethanol (absolute or 95% ethanol) to the flask and mixed, leaves the flasks at 4ºC overnight to precipitate the pullulan. After removal of the residual ethanol, the precipitate was dissolved in 10 ml of deionized water at 80ºC and the solution was dialyzed against deionized water for 48h to remove small molecules in the solution. The exopolysaccharide was precipitated again by using 20 ml of the cold ethanol and the residual ethanol was removed, the precipitate was dried at 80ºC (Moubashera and Wahshb, 2014).

Fourier transform-infrared spectroscopy (FT-IR analysis)

The crude pullulans was extracted from *Rhizopus* sp *2N* culture medium was analyzed by FT-IR spectroscopy (JASCO FT/IR). It was used under the following conditions: spectral range, 4000-400 cm^{-1} to confirm the functional groups of the extracted polymer.

Optimization of Culture Conditions for pullulans Production by *Rhizopus* sp 2N isolate

1. The type of Carbon Sources and the preferred concentration : Effect of different carbon sources on pullulans production. The selected fungal strain were grown in standard media with different carbon sources viz. Dates juice, glucose, starch, sucrose, maltose and lactose at 1 per cent level The flasks were incubated at 28°C on a rotary shaker (150 rpm) for 72h.

2. Thy type of Nitrogen Sources and the preferred concentration : Effect of media ingredients like nitrogen sources on pullulans production was determined by simply replacing nitrogen source with other nitrogen sources (peptone, yeast extract, peptone and yeast extract, Ammonium chloride, ammonium sulphate, urea) and used Dates juice Carbon Source. The flasks were incubated at 28°C on a rotary shaker (150 rpm) for 72h.

Results and Discussion

Isolation and characterization of fungal strains : A total of chance 50 fungal isolates were obtained from(Apple rotten.Olive tree leaves.River water.Soil Agricultural land) identified by Karbala University Mycological Centre as.

Pullulan was determined in fungal isolates by using alcohol precipitation (Singh *et al*., 2016). Biosynthesis of fungal glucan “pullulan” was occurred intracellularly at the cell membrane or cell wall and microorganisms secreted this homopolysaccharide out to the cell surface to form a loose and slimy layer (Ma *et al*., 2015). Its solubility in Fermentations broth is excellent as a result of the linkage patterna. In this study using rapid pre-heat treatment, dialyzed and re precipitation for the fermentation broth samples were describe as a final step to partial purity of crude pullulan. Most of the thermo-sensitive protein can be precipitated at 80°C with the time duration of 30 min by heat treatment, some of protein

Fig. 1 : Precipitation of pure and clear pullulan with cold ethanol.

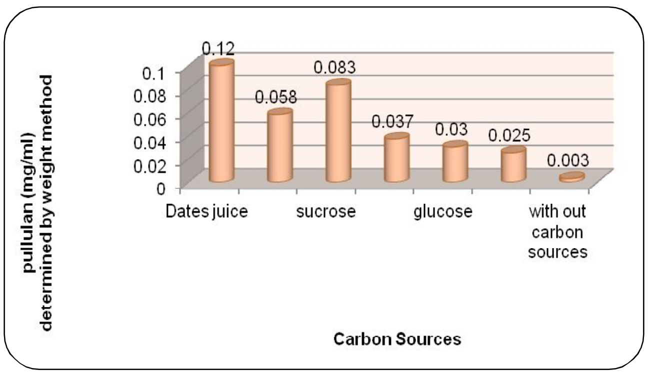

Fig. 2 : Effect of Carbon Sources in growth of *Rhizopus* spp 2N and pullulan production.

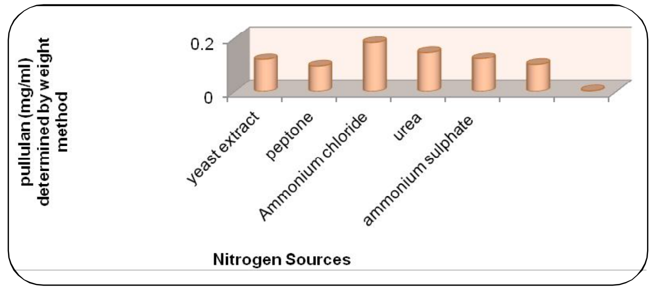

Fig. 3 : Effect of Nitrogen Sources in in growth of *Rhizopus* spp 2N and pullulan production.

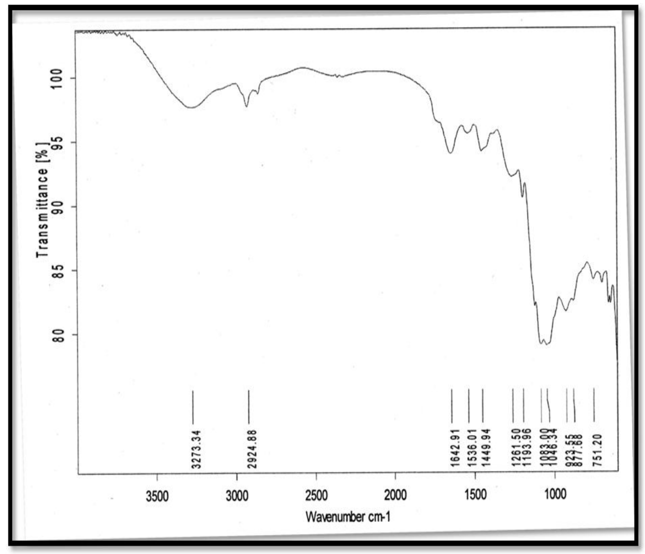

Fig. 4 : IR Spectra of pullulan produced by *Rhizopus* spp 2N from sucrose.

Taple 1 : Infra-red spectroscopy data of pullulan produced from *Rhizopus* spp 2N.

Peak location (wave number) in cm^{-1}	**Functional Group**
3293 cm^{-1}	indicated that all the pullulans had some repeating units of –OH as in sugars.
2923 cm^{-1}	indicated a SP3-hybridisation of C–H bond
1643 cm^{-1}	for the O-C-O bond
(1500–650 cm^{-1})	It is specific area to characterised the pullulan molecule
992α cm^{-1}	for the C-O bonds in the alkane compounds existed in all the samples
877 cm^{-1}	is characteristic of the α -D-glucopiranosid units
751 cm^{-1}	indicates the presence of α - (1→4)-D-glucosidic bonds
923 cm^{-1}	proved the presence of α - (1→6)-D-glucosidic bonds.

is enzymes (may be Pullulanase). After cold absolute ethanol was added to culture filtrate white polymar crystals appearing immediately and leaves overnight to Completeness of Precipitation (fig. 1).

Three fungal isolates have the ability to utilize (sucrose) carbon sources to stimulate pullulan formation is (*Aureobasidium pullulans* 1N and *Aureobasidium pullulans* 2N, *Rhizopus* spp 2N), *Rhizopus* spp 2N was bestest for production.

Although, moste studies pullulan production focuse from *A. Pullulans* (Kang *et al*., 2011; Reis *et al*., 2002), other producer strains such as *Temella mesenterica*, *Cyttaria harioti*, *Cryphonectria parasitica* and *Teloschistes flavicans* were also reported (Cheng *et al*., 2011; Chan *et al*., 2012).

The differ fungal isolates of pullulan biosynthesis depend on gene expression of the key enzymes (UDP-glucose pyrophosphorylase (UGP, EC 2.7.7.9), pullulan biosynthesis are α-phosphoglucose mutase (PGM, EC 5.4.2.2), and glucosyltransferase (FKS, EC 2.4.1.34)) to pullulan production (Wang *et al*., 2015; An *et al*., 2017).

The type of carbon source : One of the most crucial variables affecting pullulan biopolymer production is the carbon source (Oliveiraa *et al*., 2015). In this concern, the effect of different carbon sources on pullulan production by *Rhizopus* spp 2N .were investigated. The strain exhibited nutritional versality in terms of varied growth and pullulan production when tested on various carbon sources. Results in fig. 2 showed the growth and Pullulan accumulation measured after 72h incubation. Obviously, the maximum pullulan production was attained when Dates juice was used as a sole carbon source. Amount of pullulan was clearly decreased when used other source such glucose, starch, sucrose, maltose and lactose. The preferred carbon source to pullulan production was different Dependence on type of fungal strains (Singh *et al*., 2016; Ma *et al*., 2015).

Effect of Nitrogen Sources for Pullulan production

Pullulan production has been previously shown to be affected by the Nitrogen Sources used and concentration (Stankovic, 2005). In this study, six Nitrogen sources were tested. These include (peptone, yeast extract, peptone and yeast extract, Ammonium chloride, ammonium sulphate, urea. In fig. 3 showed the growth and pullulan accumulation measured after 72h incubation, Ammonium chloride is the preferred source in the pullulan Production. The synthesis of pullulan is significantly increased by the addition of various complex nitrogen sources. The importance of the presence of acetyl CoA and NADPH, a cofactor of the reductase in the pullulan synthetic pathway (Oliveiraa *et al*., 2015; Sing *et al*., 2016; Ma *et al*., 2015).

FTIR Analysis for Functional group identification

The chemical structure and functional groups of the extracted pullulan were identified by using FT-IR spectroscopy and the result obtained is exactly similar to that of other researchers (An *et al*., 2017; Me *et al*., 2014; Thirumavalavan *et al*., 2009) explain in taple 1 and fig. 4.

Conclusion

The results of the present investigation provides basis for assessing a potential for using *Rhizopus* spp. for Pullulan (a biodegradable plastic) production, which is an economically and environmentally important product, on large industrial scale, solving by this one of the problems of solid waste management that results from the accumulation of plastics and saving the environment from additional air pollution caused by its recycling and used Dates juice as renewable rawmaterials, since they were rich in carbon and nitrogen respectively, leading to develop a lowcost process of Pullulan production. FT-IR peaks give the organic structural configurations of the pullulan which is same as the standard pullulan of other researchers.

Acknowledgements

Authors are thankful to Pharmacy College, University Karbala, Holy Karbala governorate, Iraq for the support of research project. Thanks are also extended to the pharmace College Dean, Dr. Alaa Abdul Abbas Ali Aljanabi, Ahl Al Bayt University, Iraq and Dr. Alaa Abdul Hussein Al-Daamy, Applied Medical Sciences College, Karbala University, Iraq for providing the research facilities.

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