Journal of Genetic and Environmental Resources Conservation, 2015, 3(1):10-16.
www.jgerc.com

Utilization of Iraqi date syrup as substrate for production of chitosan by fungi on polyethylene sheet: 1-Optimization of production condition

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Abstract

This study was conducted to investigate the potential of Iraqi date syrup as substrate for the production of chitosan from four species of filamentous fungi named *Mucor rouxii, Aspergillus niger, Aspergillus oryzae* and *Rhizopus oryzae* in submerged fermentation. The fermentation was carried out using polyethylene sheet (PES) as a synthetic pad for growth. Out of these four fungi *Mucor rouxii* showed the maximum yield of chitosan production in different media and was used for further optimization studies included agitation speed, incubation period, initial pH of media and incubation temperature to obtain the optimum conditions to produce a maximum yield of chitosan. The results indicated the possibility of producing about 2.84g of mycelium from 100ml of date syrup with 1. 89g of chitin which is converted in to 1.10g of chitosan (38.73%) after 120hrs of cultivation at pH 6 and at 35^{0}C without agitation. Fungal chitosan was characterized with deacetylation degree 78%, a molecular weight 83kDa and their FT-IR spectra were compared with that of shrimp chitosan. These results suggest that date syrup can be used for production in an environmentally friendly way a reasonable amounts of chitosan from *Mucor rouxii* which has potential application in different field.

Keywords : Date syrup, Fungal chitosan, Polyethylene sheet, Submerged fermentation.

Introduction

Chitosan, being polycationic, non-toxic, biodegradable, biofunctional as well as a strong antimicrobial and antifungal agent (Zhang *et al.,* 2011; Yen *et al.*, 2009). It has many applications especially in a biotechnology (enzyme immobilization, chromatographic support, paper-strengthening agents and antimicrobial compounds) (Logeshet *et al.,* 2012), in food and nutrition (emulsifying, thickening and stabilizing agent, packaging membrane, antioxidant and dietary supplement), water engineering (occulants, chelating agent for metals) (Mohanasrinivasan *et al.*, 2014; Ravi, 2000) and in medical field (artificial skin, drug-delivery systems, blood anticoagulants, a component of toothpaste, hand and body creams, shampoo, lowering of serum cholesterol, cell and enzyme immobilizer, as a drug carrier, material for production of contact lenses, or eye bandages and, recently, in gene therapy too) (Nazari, 2011).

Chitosan is derived from chitin, a polysaccharide found naturally in the exoskeletons of insects, shells of crustaceans such as crab, shrimp and it, besides chitin, occurs in fungal cell walls (Hui-li *et al.,* 2004; Latha and Suresh, 2013). Chitosan is commercially produced by the deacetylation of chitin obtained from shellfish, shrimp waste and crab and lobster processing using strong alkalis at high temperatures for a long periods of time (Kucera, 2004; Khorrami *et al.,* 2012) There are problems with the seasonal supply of raw materials and the high processing costs associated with chemical conversion of the chitin to chitosan (Streit, 2009). Furthermore, the chitosans derived from such a process is heterogeneous with respect to their physio-chemical properties. However, new research has been carried out on the use of alternative sources for chitosan. The studies were focused mainly on chitosan from fungi. The production and purification of chitosan from the cell walls of fungi grown under controlled conditions offer a greater potential for more consistent products (Kumaresapillai *et al.,* 2011; Al-Hooti *et al.,* 2002; Pochanavanich and Suntornsuk, 2002). There are advantages of fungal chitosan. The molecular weight and degree of deacetylation can be controlled by selecting fungal strains or varying the

fermentation conditions. Production of fungal chitosan can be carried out by solid state or submerged fermentation using agricultural wastes (Streit *et al.,* 2009). The yield of chitosan can be enhanced by optimizing the fermentation conditions to increase the concentration of mycelia in culture or the content of chitosan in mycelia (Niederhofer and Muller, 2004). It is commercially feasible if fungal chitosan is purified from fermentation byproduct, e.g. waste mycelium. Therefore, physicochemical properties and yields of chitosan isolated directly from a fungus may be optimized by controlling fermentation and processing parameters. Fungal biomass can be produced by solid-state/substrate fermentation (SSF) and submerged fermentation (SMF) (Latha and Suresh, 2013; Maghsoodi *et al.,* 2009). Solid state fermentation (SSF) techniques are particularly suitable for the production of fungal enzymes, due to their potential advantages in manufacturing products such as high yields, low energy consumption, low environmental impact of the process, and differential expression of metabolites. However, the scale-up and optimization of SSF processes necessary for commercial production, are complex and demand intensive research (Maghsoodi and Yaghmaei, 2010).

Commonly SSF involves cultivation of filamentous fungi on natural solid substrates in which the carbon source constitutes part of their structure. During microbial growth, the fungi penetrate into, and bind tightly to, the solid substrate particle which is being degraded, and therefore, the geometric and physical–chemical characteristics of the medium change over time. These characteristics makes usually impossible to weight independently the biomass of the residual substrate. Thus, indirect growth measurements have been described in the literature, such as measurement of certain cell components, or biological activities, However all these methods have their own limitations (Muhyaddin and Chechan, 2013). Solid-state fermentation (SSF) using biologically inert material such as spong as supporting medium has been used in scientific studies (Muhyaddin and Chechan, 2013) as well as in the industries because they are cost-effective, excellent thermal insulation, long durability, flexible mechanical properties, versatility, light weight, easy to manipulate, non-toxicity, totally inert to most of the chemical use in biological experiment and recyclable. Enzymes produced on sponge are easy to separate and the mycelia covered foam pieces can be reused several times.

The aim of the present study was utilization of Iraqi date wastes as substrate for production of chitosan from different local fungal isolate under submerged fermentation using polyethylene sheet as synthetic support and determination of optimized production condition.

Materials and Methods

Fungal strains: Four strains of fungi, *Mucor rouxii*, *Aspergillus niger*, *Aspergillus oryzae* and *Rhizopus oryzae* obtained from Food Science and Biotechnology Department, University of Baghdad/ Iraq were used in this study. They were previously isolated from different lignocelluloses agriculture wastes. Potato dextrose agar (PDA) slants was prepared according to the manufacturer's instructions in order to cultivation, activation and maintenance the cultures at 4°C.

Preparation Spore Suspension: Sterile distilled water was used to wash spores from PDA slants of cultures and scraping the surface with a sterile spatula. The number of spores collected was determined with Haemocytometer. Spores in the suspension were adjusted to 5×10^7 spores/ml.

Preparation of Synthetic Support: Polyethylene sheet (PES) was used as synthetic pad for growth of fungi. It was washed several times with distilled water, dried in oven at 45°C and cut in regular pieces with a dimension $19.5\times4.4cm^2$.

Fermentation medium: The following three fermentation media were used to study the growth and production of chitosan:

a-Yeast peptone dextrose medium (YPD): contains yeast extract 0.3%, peptone 1% and dextrose 2%.

b-Molass medium (MM): Molasses was added to the media to obtain concentration 3%.

c-Date syrup media (DSM): Syrup from pitted Iraqi date named Zahdi was prepared by mixing with water in 1:3 ratio in classical mixer and equilibrated at room temperature for 5 min. The samples were irradiated by ultrasound waves with 150W intensity for 10min. After diluting the mixture with water at a ratio of 1:9 to obtain concentration 21%. , it was twice filtered; first with filter cloth (for removing date particles) and then by Millipore filter paper using a laboratory vacuum pump for cold sterilization of the date syrup. Then, the exudates fraction from the Millipore filter was used for culture media base. The pH of all media was adjusted to 6 and 100ml of each, was added to a 250ml erlenmeyer flask with a sheet of polyethylene and sterilized by autoclaving at 121°C for 15min (Nazari, 2011).

Growth and harvest of fungal mycelium: The prepared media were inoculated with 1ml of fungal spore suspension containing 5×10^7 spores and incubated at 25°C for 10 days. At the end of the desired incubation period mycelia were harvested

from polyethylene sheet and washed with distilled water until a clear filtrate was obtained. Mycelia were dried in oven.

Extraction of chitosan: To each of 1g of dried mycelium 50ml of 1N NaOH solution was added and mixed carefully. The mixture was heat treated by autoclave at 121°C for 20min. The alkali insoluble materials (AIM) were collected by centrifugation at 6000rpm for 20min, and then washed several times with distilled water until neutralized (pH 7). AIMs were dried in an oven at 40°C (Maghsoodi *et al.,* 2009). They were then treated with acetic acid 2% (v/v), as a chitosan solvent, under a reflux condition for 6hrs at 95°C (1:30 w/v). The acid insoluble fraction was separated by centrifugation at 6000rpm for 15-20min and the supernatant containing the chitosan was isolated. The pH was adjusted with a 2N NaOH solution in order to precipitate the fungal chitosan. The occulted chitosan was centrifuged at 6000rpm, for15min. Isolated chitosan was washed four to five times with distilled water to neutralize it. At the same time, ethanol (96%) and acetone were employed (1:20 w/v) to rinse the chitosan and then it was dried in a vacuum oven dryer at 60°C (Maghsoodi *et al.,* 2009).

Optimization studies on selected fungi: Optimization studies were carried out by changing a single parameter at a time during chitosan production. The different parameters studied include agitation, incubation period, pH, different fungal medium, temperature, and polyethylene sheet as synthetic pad for growth. Effect of synthetic pad was studied by using one set of flasks with polyethylene sheet and another set without polyethylene sheet. So was done with agitation at a speed 150 rpm. The effect of incubation period was studied by estimation of chitosan produced at 72,96,120,144 and 168 hrs. The effect of pH was studied by adjusting the initial pH of date syrup media to 2, 4, 6, 8 and 10.

Characteristics of chitosan:

Degree of deacetylation(DD): Infrared spectrum of fungal chitosan was used to monitor the chitosan extraction by comparing it with the standard spectrum of Sigma chitosan. The transmittance was carried out in the form of KBr pellets in the range of 400-4000cm^{-1} using infra-red spectrophotometer (FT-IR).The degree of deacetylation (DD) of chitosan samples and commercial chitosan was calculated using the equation proposed by Maghsoodi *et al.* (2009):DD = 100-[(A1655/A3450 × 100/ 1.33]

A1655 and A3450 are the absorbance respectively at1655 and 3450cm^{-1}

Determination of viscosity: The viscosity of 1% chitosan in 2% acetic acid solution was determined using an Ostwald viscometer at 25ºC.

Determination of molecular weight: The average molecular weight of chitosan was determined according to the viscometric method. The average molecular weight was then calculated using the Mark-Houwink equation. Mark-Houwink equation, $\eta = k\,Mv$ where η is the intrinsic viscosity using the constants $K = 1.81x10^{-5}$ and $a = 0.93$, K and a are coefficient related to the Ubbelohde tube a the molecular weight of sample, *Mv* is the viscosity average (Roberts, 1992)

Results and Discussion

The results in the Table (1) shows that different yields of mycelium and chitosan were obtained from different strains of fungi used in this study when they cultivated in yeast peptone dextrose medium (YPD) for 10 days with or without polyethylene sheet as synthetic pad for growth. It was found that the mycelium yield *of Mucor rouxii* was 1.80gm/100ml of media followed by *Aspergillus niger, Aspergillus oryzae* and *Rhizopus oryzae* which were produced 1.68, 1.00 and 0.80gm of mycelium respectively. Whereas, the maximum yield of mycelium under the same condition but without polyethylene by *Mucor rouxi* and *Aspergillus niger* was 1.00gm and less than that for other two remained strains. Chitin was prepared after a hot alkaline treatment dried and the dry weight was determined. The dry weights of the chitin extracted are presented in the Table (1). The maximum yield 600mg of chitin was obtained from *Mucor rouxii*, mycelium which represented about 33.3% (Figure1). Lath and Suresh (2013), observed that typical yields of chitin after 120hrs growth were generally in the range of 53% of the dry weight of the mycelia form of *M. rouxii*.

The present study showed that 60% of chitin could be obtained after 5 days of incubation under submerged fermentation with polyethylene (PES) as pad for fungal growth. This increase can be attributed to the time dependant increase in chitin content of cell wall of *Mucor rouxii*. The dry weight of chitosan was determined and presented in the Table(1). *Mucor rouxii*. produced maximum amount of chitosan, which was 324mg of mycelium on a dry weight basis, followed by *Rhizopus oryzae* which produced 180mg. Lath and suresh (2013) observed that 280.5mg chitosan could be producted from 1g of mycelium using *Mucor* sp. Among the selected four fungal strains, *Mucor rouxii* showed the maximum yield of chitosan under submerged fermentation with polyethylene (PES) from its fungal mycelium and *Aspergillus oryzae* showed the minimum yield. Hence, *Mucor rouxii* was selected for the further optimization studies.

Optimization studies:

Growth medium: The results shown in Table (2) clearly indicated that date syrup could be an excellent medium for production of chitosan. The yield of chitosan obtained from *Mucor rouxii* grown on date syrup was 680mg of mycelia obtained, while it was 324 and 631mg when YPD and MM where used as media for growth. The date syrup mainly contains carbohydrates with small amount of fats and proteins along with micro and macroelements (Al-Farsi *et al.,* 2007; Al-Hooti *et al.,* 2002) The effectiveness of date syrup for production of chitosan may also be due to the presence of fibrous material which increase the interparticle spacing for aeration and diffusion of nutrient In view of its effectiveness and low cost, date syrup was chosen as the best medium for maximum chitosan production under submerged fermentation with polyethylene (PES) for farther study.

pH: The effect of pH on chitosan production was studied by determination of chitosan yield at different pH values ranged from 2 to 8. The fungal growth was carried out under submerged fermentation with polyethylene (PES) using date syrup as media for 120hrs.

The maximum chitosan production was observed (699 mg) at pH 6 and it was found that any deviation from this value towards the increase or decrease will affects the production of mycelia and chitosan negatively (Table 3).

Temperature: As shown in Table (4) it was found that 35ºC is the optimum temperature for mycelium and chitosan production by *Mucor rouxii* from date syrup. The amount of dry mycelium obtained in this temperature was 2.133g containing 1362mg of chitin can be extracted and converted in to 801mg of chitosan. The total yield was 37.55% of dry mycelial mass. The optimized yield of chitosan from *Mucor rouxii.* is much more than previously reported values. Latha and Suresh (2013) reported 31.17% chitosan yield from dry mycelium.

Incubation period: The yield of mycelium, chitin and chitosan produced by *Mucor rouxii* grown on date syrup was studied for an interval incubation period hold on 168hrs. (7 days) and it was found that the maximum yield of mycelium (2.114gm/100ml of media) and chitosan (794mg) could be obtained after 120hrs. A light deterioration of production was observed by elongation the incubation time. The yield of mycelium and chitosan was declined to 2.011gm/ 100ml and 755mg after 148hrs. respectively. More deterioration was observed by elongation the time of incubation as shown in Table (5). It was previously reported that the mycelium yield increased with time and reached a maximum after 144hrs of growth (Latha and Suresh, 2013).

Agitation: The results presented in Table 6 shown that the maximum chitosan yield was observed in a medium without agitation. 2.844g of mycelium was produced on date syrup which found to be contains 1899 mg of chitin, which converted in to 1101mg of Chitosan. Whereas the chitosan yield with agitation was 797mg. Hence further studies were carried out without agitation.

The results observed in the present study are in contrary to the previous reports that agitation resulted in decrease in dry mycelium mass. This controversy may be due to strain differences.

The physical appearance of the produced chitosan was pale, white and flaky. Different types of chitosan were available in market such as: high molecular weight chitosan, low molecular weight chitosan, water-soluble chitosan, pharm grade chitosan, high density chitosan and chitosan oligosaccharide. These types of chitosan have different solubility.

In our study, the produced fungal chitosan was insoluble in water and alkali solutions but soluble in organic acid (99.8% in 5% acetic acid). The molecular weight was 83KDa and degree of deacetylation of fungal chitosan was78%, relatively lower than that of crab chitosan. Results were slightly different from the reported percentage degrees of deacetylation of chitosan from other fungi (Ali *et al.,* 2013). The degree of deacetylation is an important parameter affecting of physico-chemical properties of chitosan. In fact, the large positive charge density due to the high degree of deacetylation makes fungal chitosan unique for industrial applications, particularly as a coagulation agent in physical and chemical waste-treatment systems, as a chelating and clarifying agent in the food industry, and as an antimicrobial agent.

Figure(1): Prepared chitosan (right) before drying and (left) chitosan flakes after drying and grinding

Table (1): Production of chitosan from different fungal species

Fungal species	Synthetic Support (PES)	Product (Dry weight)		
		Mycelium g/100ml of media	Chitin (mg)	Chitosan (mg)
Mucor rouxii	without	1.00	400	212
	with	1.80	600	324
Aspergillus niger	without	1.00	280	70
	with	1.68	320	162
Aspergillus oryzae	without	0.38	199	38
	with	1.00	251	48
Rhizopus oryzae	without	0.50	395	58
	with	0.80	450	180

Table (2): Effect of type of growth medium on mycelia dry weight and chitosan production

Growth Medium	Product (Dry weight)		
	Mycelium g/100ml of media	Chitin (mg)	Chitosan (mg)
YPD	1.8	600	324
MM	1.95	1189	631
DSM	2.012	1251	680

Table (3): Effect of pH of growth media on mycelia dry weight and chitosan production

pH	Product (Dry weight)		
	Mycelium g/100 ml of DSM	Chitin (mg)	Chitosan (mg)
pH2	0.88	592	22
pH4	1.58	1129	422
pH6	2.012	1259	699
pH8	0.801	448	222

Table (4):Effect of temperature on mycelia dry weight and chitosan production

Temperature	Product (Dry weight)		
	Mycellium g/100ml of DSM	Chitin (mg)	Chitosan (mg)
25	2.012	1251	680
30	2.063	1289	701
35	2.133	1362	801
40	2.081	1209	704
45	1.899	1189	531

Table (5): Effect of incubation period on mycelia dry weight and chitosan production

Incubation period (hrs)	Product (Dry weight) Mycellium g/100ml of DSM	Chitin (mg)	Chitosan (mg)
72	0.88	591	233
96	1.981	1199	653
120	2.114	1362	794
148	2.011	1201	755
168	1.999	991	601

Table (6): Effect of agitation on chitosan and mycelia dry weight yield in submerged fermentation with polyethylene sheet

Agitation	Product (Dry weight) Mycellium g/100 ml of DSM	Chitin (mg)	Chitosan (mg)
Without agitation	2.844	1899	1101
With agitation	2.114	1363	797

Conclusions

The present study conclusively showed that 38.71% of dry mycelium can be converted in to chitosan under optimal conditions had deacetylation degree of 78%, a molecular weight 83kDa and their FT-IR spectra were compared with that of shrimp chitosan. Commercial chitosan could be obtained from *Mucor rouxii* mycelia and would have potential application for medical and agricultural uses.

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