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Chitosan production by psychrotolerant *Rhizopus oryzae* in non-sterile open fermentation conditions

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ABSTRACT

A new chitosan producing fungus was locally isolated from soil samples collected around Erzurum, Turkey and identified as *Rhizopus oryzae* PAS 17 (GenBank accession number KU318422.1). Cultivation in low cost non-sterile conditions was achieved by exploiting its ability to grow at low temperature and pH, thus, undesired microbial contamination could be eliminated when appropriate culture conditions (incubation temperature as 15 °C and initial pH of the medium as 4.5) were selected. Medium composition and culture conditions were optimized using Taguchi orthogonal array (OA) design of experiment (DOE). An OA layout of L16 (4^5) was constructed with five most influensive factors at four levels on chitosan production like, carbon source (molasses), metal ion (Mg^{2+}), inoculum amount, agitation speed and incubation time. The optimal combinations of factors (molasses, 70 ml/l; $MgSO_4 \cdot 7H_2O$, 0.5 g/l; inoculum, 6.7×10^6 spores/disc; agitation speed, 150 rpm and incubation time, 8 days) obtained from the proposed DOE methodology was further validated by analysis of variance (ANOVA) test and the results revealed the increment of chitosan and biomass yields of 14.45 and 8.58 folds from its unoptimized condition, respectively.

1. Introduction

Chitosan is a linear polymer composed β-1,4-linked D-glucosamine residues with various degrees of *N*-acetylated residues and is obtained by chemical deacetylation of chitin that is the second most abundant polysaccharide in the nature after cellulose. Chitin exists in the waste exoskeletons of crustacean, insects, crab, shrimp and lobster [1,2]. Chitosan has an important role in various industrial applications such as food, medicine, environmental protection, etc. Use of chitosan is going to increase day-by-day due to its excellent compliance with demands of the present day. For example it is used in edible films and coatings, tissue engineering, elimination of environmental pollutants and so many other applications [3–7]. Commercial chitosan production, is carried out by the waste exoskeletons of the animals named above, that has many problems such as limited and seasonal supply of raw materials, production sustainability, hot alkali processing cost and inconsistent quality characters. Besides chitosan can be produced using series of some processes containing demineralization, deproteinization, decolourisation and deacetylation. On the other hand, the cell walls of filamentous fungi, especially class zygomycetes, present an alternative and effective chitosan source due to its easy handling and harvesting, manipulation properties on molecular weight, and better solubility. Furthermore this class of fungi contains chitin, proteins, polyphosphates and polyglucuronic acid with chitosan in their cell walls [2,8]. Some of physicochemical characteristics such as molecular weight, more consistent property and degree of deacetylation of fungal chitosan can be controlled and varied by manipulations on environmental and nutritional conditions compared to chitosan obtained from crustacean sources [9]. Microbiologically produced chitosan by fungi presents numerous applications in food, agriculture, pharmaceutical industries due to its biodegradable structure [10,11].

In the last decades, it has widely been studied on reduction of the harmful effects of some additives and protective materials that cause human hood health problems in the long term, used in the food industry, medicine and several industrial production processes. Besides, there is an increasing demand to reduce production cost in various industries. Preliminary studies are carried out in laboratories with low cost, but when they are carried out in industrial scale, a huge production cost can occur. Therefore, in order to reduce production cost and save time and money alternative fermentation conditions are developed. Use of waste materials or by-products, that's minimal nutrient consumption provide cost

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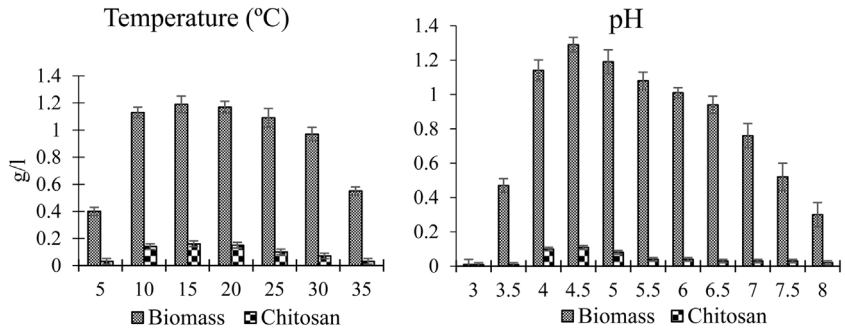

Fig. 1. Optimization of fermentation temperature and pH by OVAT method[1,2]. Error bars mean ± SD.
[1]Medium composition for temperature: 30 ml/l molasses, 3 g/l $(NH_4)_2SO_4$; 1.5 g/l KH_2PO_4, 0.1 g/l $MgSO_4{\cdot}7H_2O$; 0.02 g/l $FeSO_4{\cdot}7H_2O$.
Fermentation conditions: pH 5.0, agitation 100 rpm and incubation time 4 days.
[2]Medium composition for pH: 30 ml/l molasses, 3 g/l $(NH_4)_2SO_4$; 1.5 g/l KH_2PO_4, 0.1 g/l $MgSO_4{\cdot}7H_2O$; 0.02 g/l $FeSO_4{\cdot}7H_2O$.
Fermentation conditions: temperature 15 °C, agitation 100 rpm and incubation time 4 days.

effective production process. As usual, sterile culture conditions were used for microbial fermentation processes in many reports [12–16]. On the other hand, use of non-sterile conditions can be preferred instead of sterile conditions to reduce production cost and labor [17]. Besides this, some disadvantages occur in non-sterile culture conditions as undesirable microbial contaminations and reduction of the yield [18]. Hence, these situations should be reduced as soon as possible by some manipulations. In the current study, the optimization was carried out at two steps. First step was consisted of the incubation temperature and pH of the medium adjustment by traditional OVAT method. The purpose of this was to minimize microbial contaminations and to maximize the growth conditions of *R. oryzae* PAS17. In the next step Taguchi orthogonal array (OA) design was employed to optimize the other effective factors for chitosan production. Use of statistical experimental design methods are useful tools when compared with the classical OVAT method [19]. Taguchi design of experiment (DOE) method is conventionally used for offline quality control and provides the combination of experimental design techniques with quality loss consideration.

Chitosan production by chemical pathway requires expensive methods, and use of acid and alkali chemicals exists waste problems. *Rhizopus*, a genus of zygomycetes fungi, is used for effective chitosan production [1,10,20], and there are many reports about non-sterile conditions [21,22] however there is not any paper about chitosan production by psychrotolerant *Rhizopus oryzae* in non-sterile conditions, unless stated otherwise.

2. Materials and methods

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Molasses was obtained from local Erzurum Sugar Factory (Erzurum, Turkey). The medium used for chitosan production was consisted of the following (g/l): 3 $(NH_4)_2SO_4$, 1.5 KH_2PO_4, 0.02 $FeSO_4{\cdot}7H_2O$. Molasses and $MgSO_4{\cdot}7H_2O$ were added at different concentrations ranged between % 3–9 and 0,1–0,7 (g/l), respectively. 100 ml medium was used as the working volume in 250 ml Erlenmeyer flasks in all experiments.

2.1. Isolation and identification of microorganism

R. oryzae strains were isolated from various soil samples that collected around Erzurum. Briefly, 1 g of soil sample was suspended in 10 ml sterile-saline water. The obtained suspension was diluted up to 10^{-3} with sterile-saline water. One ml of the suspension was plated on petri dish containing 20 ml of molasses agar medium. In order to prepare this agar medium, 20 g agar was dissolved in 1 l molasses solution containing 3% of molasses. The pH of the medium was adjusted to 5.0. Inoculated petri dishes were incubated at 10 °C for 15 days. At the end of incubation period, colonies having well growth were subcultured and purified. The strain named PAS17 showed maximum growth and chitosan production, thus, it was used in the subsequent experiments. Taxonomic identification of the most effective isolate was made using mature cultures on standard potato dextrose agar plates at 10 °C in order to ensure a good development of taxonomic relevant features and key structures [23,24]. Molecular identification of PAS17 was made using 18S rRNA sequence analysis technique (GenBank accession number KU318422.1).

2.2. Fungal biomass production

At the end of the incubation period, the biomass of *R. oryzae* PAS17 was collected. Obtained biomass was harvested on a screen, washed with distilled water for 3 times and dried in an oven at 40 °C to a constant weight.

2.3. Preparation of alkali insoluble material (AIM)

To remove protein residues, the dried biomass was treated with 0,5 M NaOH (30 ml/g) at 121 °C for 20 min [2]. Alkali insoluble material (AIM) was separated from the mixture by centrifugation (15 min, 5000*g*) and washed three times with distilled water. AIM was dried in an oven at 40 °C to a constant weight and stored at 4 °C until use.

Table 1
Selected culture conditions and assigned levels.

Serial No.	Factors	Level 1	Level 2	Level 3	Level 4
1	Molasses (ml/l)	30	50	70	90
2	$MgSO_4{\cdot}7H_2O$ (g/l)	0.1	0.3	0.5	0.7
3	Inoculum (mm diameter)	4	6	8	10
4	Agitation (rpm)	100	150	200	250
5	Incubation time (d)	4	6	8	10