Carbohydrate Polymers 83 (2011) 1330–1337

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Optimization of pullulan production from hydrolysed potato starch waste by response surface methodology

Yekta Göksungur[a,*], Pürlen Uzunoğulları[a], Seval Dağbağlı[b]

[a] Ege University, Faculty of Engineering, Department of Food Engineering, 35100 Bornova, İzmir, Turkey
[b] Celal Bayar University, Faculty of Engineering, Department of Food Engineering, 45140 Muradiye, Manisa, Turkey

ARTICLE INFO

Article history:
Received 5 May 2010
Accepted 24 September 2010
Available online 25 October 2010

Keywords:
Pullulan
Polysaccharide
Calcium alginate
Immobilization
A. pullulans
Response surface methodology
Amyloglucosidase
Pullulanase

ABSTRACT

The production of pullulan from hydrolysed potato starch waste by *Aureobasidium pullulans* P56 was investigated. The liquefaction of potato starch was done by Ca-alginate immobilized amyloglucosidase and pullulanase enzymes in a packed bed bioreactor. Various organic nitrogen sources were tested and none of the nitrogen sources gave pullulan concentrations as high as that obtained with yeast extract. Response surface methodology was used to investigate the effects of three factors (incubation time, initial substrate concentration and initial pH) on the concentration of pullulan in batch cultures of *A. pullulans*. No previous work has used statistical analysis on the optimization of process parameters in pullulan production from hydrolysed potato starch waste. Maximum pullulan concentration of 19.2 g/l was obtained at the optimum levels of process variables (incubation time 111.8 h, initial substrate concentration 79.4 g/l, initial pH 7.26). The optimization led to a 20% increase in pullulan concentration.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

Pullulan is an extracellular water-soluble microbial polysaccharide produced by strains of *Aureobasidium pullulans*. It is a linear mixed linkage α-D-glucan consisting mainly of maltotriose units interconnected via α-(1→6) linkages (Roukas, 1998). Typical industrial uses of pullulan are as food coatings and packaging material due to its good film-forming properties, as an ingredient of low calorie foods and as a starch substitute; as films, with properties similar to polyvinyl alcohol, but superior in many ways as well as being biodegradable; as an adhesive in the form of pastes with water, as a construction material (after esterification) with fibers similar in strength and elasticity to those in nylon; and as a bulking agent and stabilizer for tablets in the pharmaceutical industry (Israilides, Scanlon, Smith, Harding, & Jumel, 1994).

The cost of pullulan production currently is relatively high and therefore, it is prudent to search for inexpensive carbon and nitrogen sources, which are nutritionally rich enough to support the growth of the microorganism as well as the production of pullulan (Wu, Jin, Tong & Chen, 2009). There are various reports on the production of pullulan from different sources such as sweet potato (Wu et al., 2009), potato starch waste (Barnett, Smith, Scanlon & Israilides, 1999), coconut by-products (Thirumavalavan, Manikkadan & Dhanasekar, 2009), deproteinized whey (Roukas, 1999a), agro-industrial wastes such as grape skin pulp extract, starch waste, olive oil waste effluents and beet molasses (Israilides et al., 1994), brewery wastes (Roukas, 1999b), beet molasses (Roukas, 1998), jaggery which is a concentrated sugar cane juice (Vijayendra, Bansal, Prasad & Nand, 2001), carob pod (Roukas & Biliaderis, 1995) and Jerusalem artichoke (Shin, Kim, Lee, Cho & Byun, 1989).

If starch is used as substrate for pullulan production, it needs to be hydrolysed since *A. pullulans* cannot use native starch molecules. Starch consists of two molecules: amylose and amylopectin. Amylose is formed from glucose units linked by α-1,4 and amylopectin is formed from α-1,4 linked chains of glucose with α-1,6 linked branch points. A number of enzymes are used in starch hydrolysis. α-Amylase is used to pre-thin the gelatinized starch and hydrolyses α-1,4 linked glucose units to low molecular weight dextrins in the liquefaction step. Pullulanase hydrolyses α-1,6 branch links while glucoamylase hydrolyses α-1,4 links releasing glucose units from the non-reducing end of the chain. The α-1,6 branch links are also hydrolysed by glucoamylase but much less rapidly. For this reason, it is advisable to supplement glucoamylase with a debranching enzyme, pullulanase, which hydrolyses α-1,6 branch links in the amylopectin molecule (Roy & Gupta, 2004).

Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effect of factors and searching optimum conditions for desirable responses. The conventional practice which varies one variable at

* Corresponding author. Tel.: +90 232 3882395; fax: +90 232 3427592.
E-mail address: yekta.goksungur@ege.edu.tr (Y. Göksungur).

0144-8617/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2010.09.047

a time, does not allow evaluation of the combined effects of all the factors involved in the process and constitutes a time consuming methodology. These restrictions can be overcome by the use of RSM which can identify and quantify the various interactions among different parameters. RSM has been extensively used to optimize the cultural medium conditions and other parameters in bioprocesses (Li, Bai, Cai & Ouyang, 2002; Ürküt, Dağbağlı & Göksungur, 2007). In the previous investigation of our group (Ürküt et al., 2007), pullulan production using Ca-alginate immobilized *A. pullulans* cells was optimized by RSM and the maximum pullulan concentration of 21.07 g/l was obtained at the optimum concentrations of process variables (pH 7.31, agitation speed 191.5 rpm, incubation time 101.2 h). Our earlier report (Göksungur, Dağbağlı, Uçan & Güvenç, 2005), described the optimization of pullulan production in a stirred tank bioreactor and aeration rate, agitation speed and initial substrate concentration were found to have a strong linear effect on pullulan production. Singh et al. (2009) studied optimization of pullulan production by *A. pullulans* in batch culture using central composite rotatable design, where the simultaneous effect of five independent variables (sucrose, ammonium sulphate, yeast extract, dipotassium hydrogen phosphate and sodium chloride) were investigated. Lin et al. (2007) investigated the effects of six physical, chemical and biological factors and their interactions on the fermentation of exopolysaccharides by *A. pullulans* by using a 2-level fractional factorial design. Jiang (2010) used a synthetic medium containing sucrose and studied optimization of fermentation conditions such as temperature, fermentation time and initial pH for pullulan production by *A. pullulans* using RSM. No previous work has used RSM or other statistical techniques to optimize pullulan production from waste materials.

The present study examined pullulan production from potato starch waste by *A. pullulans* P56. The potato starch waste was first liquefied by α-amylase enzyme and then hydrolysed by Ca-alginate immobilized amyloglucosidase and pullulanase enzymes. The hydrolysate was used as substrate for pullulan production by *A. pullulans*. RSM was used to optimize fermentation parameters to obtain maximum pullulan production. Three factors (incubation time, initial substrate concentration and initial pH) considered to have significant impact on pullulan production were selected as parameters for optimization studies. This study is the first detailed work about the use of response surface methodology for the optimization of fermentation parameters in pullulan production using waste substance as substrate.

2. Materials and methods

2.1. Microorganism and culture conditions

A. pullulans P56, a strain deficient in melanin production, was supplied by Prof. Dr. T. Roukas of the Aristotle University of Thessaloniki. The microorganism was maintained on potato dextrose agar slants at 4 °C and subcultured every 3 weeks. Cells for inoculation of the culture medium were obtained from cultures grown on potato dextrose agar slants at 28 °C for 48 h. Two loops of *A. pullulans* cells were transferred to 250 ml conical flasks containing 50 ml of culture medium (pH 5.5) of the following composition (g/l): sucrose 30.0, $(NH_4)_2SO_4$ 0.6, yeast extract 0.4, K_2HPO_4 5.0, $MgSO_4{\cdot}7H_2O$ 0.2 and NaCl 1.0. The flasks were incubated at 28 °C for 48 h in a rotary shaker incubator (Gerhardt Laboshake LS 2/5 RO 2/5, Bonn, Germany) at 200 rpm. These cultures at a level of 5% (v/v) were then used to inoculate the production medium containing hydrolysed potato waste.

2.2. Media

Potato starch substrate (supplied by Özgörkey Gıda Ürünleri San. ve Tic. A.Ş., İzmir) was a waste product of frozen French fries production. The waste was dried in drum drier. Moisture (7.0%), ash (0.38%), protein (0.98%) and starch (93.0%) content of the dried samples were determined according to the official procedures 925.10, 923.03, 920.87, and 945.37, respectively (AOAC, 1999). All reagents were of analytical grade. Na-alginate used for the immobilization of Dextrozyme DX enzyme was obtained from brown algea (Sigma, A-2033, St. Louis, USA).

2.3. Enzyme immobilization

Dextrozyme DX and Liquozyme Supra enzymes supplied by Novozymes A/S (Denmark) were used for the hydrolysis of potato starch waste. Dextrozyme DX is a mixture of amyloglucosidase from *Bacillus subtilis* and pullulanase from *Aspergillus niger*. The declared amyloglucosidase and pullulanase activities were 170 AGU/g (AGU is the amount of enzyme which hydrolyses 1 μmol of maltose per minute under specific conditions) and 340 NPUN/g (one NPUN-new pullulanase unit novo-represents the endo-pullulanase activity and measured relative to a Novozymes A/S Promozyme D standard), respectively. Liquozyme Supra is a bacterial α-amylase enzyme from *Bacillus licheniformis* with a declared activity of 135 KNU/g (KNU, kilo novo units – the amount of enzyme which breaks down 5.26 g of starch per hour according to Novozyme's standard method for the determination of α-amylases).

Dextrozyme DX (12 ml, 2400 AGU + 4800 NPUN) was mixed with 150 ml of 2% Na-alginate (Sigma, A-2033) solution and stirred till a homogenous solution was formed. This was then dropped in a 2% (w/v) $CaCl_2$ solution using a peristaltic pump. Alginate drops solidified upon contact with $CaCl_2$, forming beads and thus entrapping enzymes. The beads were allowed to harden for 30 min. The beads were then washed with sterile physiological solution to remove excess calcium ions and kept at 4 °C till further use.

2.4. Hydrolysis of potato starch waste

A slurry containing 13% of starch was prepared from dried potato waste and the pH was adjusted to 5.5 by using 3 N H_2SO_4. Liquefaction was carried out at 90 °C for 90 min in the presence of α-amylase enzyme (Liquozyme Supra, 0.4 g/kg starch). After liquefaction, the temperature was quickly reduced to 60 °C and the pH adjusted to 4.3. Hydrolysis of starch was carried out using a jacketed Pyrex column packed with 2.0–2.4 mm diameter Ca-alginate beads with entrapped pullulanase and glucoamylase enzymes (Dextrozyme DX). The characteristics of the packed-bed bioreactor are given in Table 1. Prior to use, the packed-bed bioreactor was filled with Ca-alginate beads. The liquefied starch slurry was fed (0.42 ml/min) to the bottom of the column continuously by means of a peristaltic pump (Chemap AG) through silicon tubing. Effluent liquid overflowed from an outlet port at the top of the column, maintaining a constant level inside the column. The beads were trapped inside the bioreactor by a metal mesh filter covered with a plastic barrier. The temperature of the bioreactor was maintained at 60 °C by circulating water at a constant temperature from a circulator bath through the jacket of the column. Starch hydrolysis was monitored by mea-

Table 1
Packed bed bioreactor characteristics.

Packing	Ca-alginate beads
Inner diameter	1.57 cm
Column height	49 cm
Total reactor volume	95 ml
Bed volume	85 ml
Void volume	22 ml
Packing weight	67 g

Table 2
Levels of factors used in the experimental design.

Factor	Name	Level		
		−1	0	+1
X_1	Time (h)	72	96	120
X_2	Initial sugar concentration (g/l)	50	70	90
X_3	pH	6.5	7.5	8.5

suring reducing sugars in the effluent liquid overflowing from the column.

2.5. Fermentation conditions

The production medium was prepared by replacing sucrose in cultivation medium with glucose from hydrolysed potato starch waste. The production medium used had the following composition (g/l): glucose from hydrolysed starch waste 30.0–90.0, $(NH_4)_2SO_4$ 0.6, yeast extract 0.4, K_2HPO_4 5.0, $MgSO_4{\cdot}7H_2O$ 0.2 and NaCl 1.0 (pH 7.5). After adjusting the pH to 7.5 with 1 N NaOH, the substrate was sterilized at 121 °C for 20 min. Fermentations were carried out batchwise in 250 ml flasks with 50 ml working volume in a rotary shaker incubator (Gerhardt Laboshake LS 2/5 RO 2/5, Bonn, Germany) operated at 200 rpm and 28 °C. Initial glucose concentrations of 30, 50, 70 and 90 g/l were used to determine the effect of substrate concentration on pullulan and polysaccharide production. Alternative nitrogen sources (soy protein, urea, peptone, malt sprouts, tryptone and corn steep liquor) were substituted with yeast extract (0.4 g/l) on equal nitrogen bases to determine the effect of different nitrogen sources on pullulan and polysaccharide production. The optimization studies were done by using RSM and the levels of incubation time, initial substrate concentration and initial pH are given in Table 2.

2.6. Analytical techniques

The degree of hydrolysis of potato waste was measured as an increase in the content of reducing sugars. Dextrose equivalent (DE) represents the percentage of hydrolysis of the glycosidic linkages present in starch and calculated as the ratio of reducing sugars (expressed as glucose) to total carbohydrates (Khatoon, Sreerama, Raghavendra, Bhattacharya & Bhat, 2009). Total carbohydrate content of the potato waste and reducing sugar content of hydrolysed potato waste were assayed by phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956) and dinitrosalicylic acid method (Miller, 1959), respectively.

Polysaccharide levels were determined by centrifuging the fermentation medium at 4000 × *g* for 10 min and precipitating the polysaccharide with 2 volumes of ethanol at 4 °C for 1 h. The precipitate was centrifuged at 4000 × *g* for 10 min followed by drying at 80 °C overnight and then weighed. To determine the pullulan content of the polysaccharide, the precipitate was resuspended in 0.05 M sodium acetate (pH 5.0) at a concentration of 10 mg/ml. 10 μl of pullulanase (Promozyme D2, Novozymes A/S, Denmark) was added to 1 ml of this sample. The mixture was incubated at 25 °C for 21 h according to the procedure of Leathers et al. (1998). The enzyme was also added to a pure sample of pullulan (Hayashibara Biochemical Co., Japan) of the same concentration as described earlier. The glucose reducing equivalents were determined by using dinitrosalicylic acid method (Miller, 1959) and the actual pullulan content was derived. Residual sugar in the fermentation medium was determined according to dinitrosalicylic acid method (Miller, 1959) using glucose as the standard. The data reported are the average values ± SD of three replicate experiments.

Table 3
Experimental design.

Run	Time (h)	Initial sugar concentration (g/l)	pH	Pullulan (g/l)
1	120	90	6.5	13.950
2	120	70	7.5	15.920
3	120	50	8.5	9.600
4	120	90	8.5	13.732
5	120	50	6.5	12.022
6	96	70	7.5	16.000
7	96	90	7.5	15.800
8	96	70	7.5	16.100
9	96	70	6.5	15.910
10	96	70	7.5	16.100
11	96	70	7.5	16.440
12	96	50	7.5	11.517
13	96	70	7.5	15.760
14	96	70	8.5	13.587
15	96	70	7.5	15.970
16	72	90	6.5	12.897
17	72	50	8.5	5.600
18	72	90	8.5	9.832
19	72	50	6.5	9.802
20	72	70	7.5	13.620

2.7. Experimental design and statistical analysis

The statistical analysis of the data was performed using Minitab Statistical Software (Release 13.20). Details of response surface methodology can be found elsewhere (Myers and Montogomery, 1995). The levels of factors used in the experimental design are listed in Table 2. The data of the factors were chosen after a series of preliminary experiments. Twenty experiments were conducted using a face central composite statistical design ($\alpha = 1$) for the study of three factors each at three levels (Table 3). The levels were −1, 0, +1 where 0 corresponded to central point. The actual level of the central point of each factor was calculated using the following equation (Myers and Montogomery, 1995).

$$\text{coded value} = \frac{\text{actual level} - (\text{high level} + \text{low level})/2}{(\text{high level} - \text{low level})/2}$$

Response surface model was fitted to the response variable, namely pullulan concentration (g/l). The second order response function for three quantitative factors is given by Eq. (1):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

where X_1, X_2 and X_3 represent the levels of the factors according to Table 1 and $\beta_0, \beta_1, \ldots, \beta_{23}$ represent coefficient estimates with β_0 having the role of a scaling constant.

3. Results and discussion

3.1. Enzymatic hydrolysis of starch

Hydrolysis of soluble starch liquefied by α-amylase (Liquozyme Supra, Novozymes A/S) was done by using Ca-alginate immobilized pullulanase and amyloglucosidase enzymes (Dextrozyme DX, Novozymes A/S) in a packed bed bioreactor. The pH of a slurry of dried potato waste at the concentration of 13% was adjusted to pH 5.5 for liquefaction. The liquefaction process was done at 90 °C for 90 min with the addition of α-amylase enzyme (0.4 g/kg starch) and liquefied starch with DE of 38.2 was obtained. The liquefied starch was then loaded to a packed bed of Ca-alginate immobilized Dextrozyme DX which is a blend of pullulanase and glucoamylase activities, at a flow rate of 0.42 ml/min. A syrup with 12.2% glucose (DE 95.9) was obtained in the effluent liquid overflowing from the packed bed bioreactor.

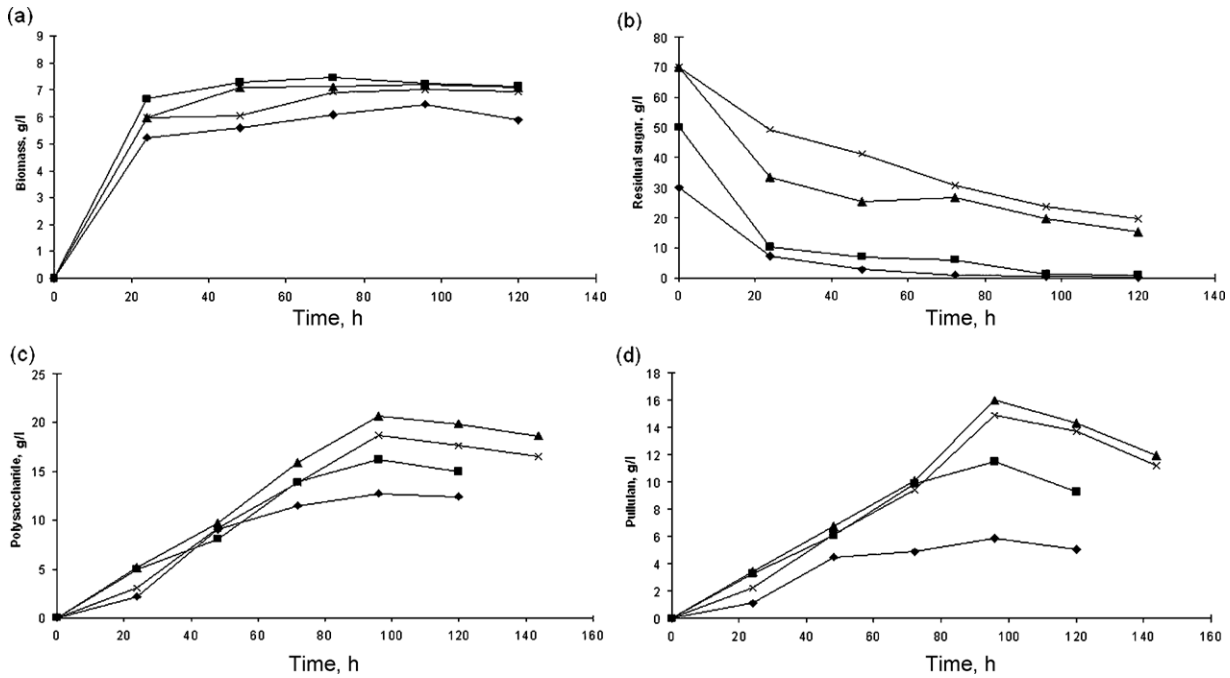

Fig. 1. Kinetics of pullulan, polysaccharide and biomass production together with sugar consumption for fermentation media containing 30 (♦), 50 (■), 70 (▲) and 90 g/l (×) of initial sugars (28 °C, pH 7.5, the standard deviation of each experimental point ranged from 1.6 to 3.6).

The entrapment in calcium alginate gel beads is one of the most widely used methods for cell immobilization owing to its simplicity, non-toxicity, mild gelation conditions and ease of use (Göksungur, Gündüz & Harsa, 2005). However, calcium alginate beads are normally not used for enzyme entrapment as large pore sizes of these beads result in enzyme leakage out of such beads. High DE value (DE 95.9) obtained in this study proved that glucoamylase and pullulanase were bound to alginate and this combination of binding and entrapment hold the enzymes inside the beads. Similar result was found by Roy and Gupta (2004) who studied hydrolysis of potato starch by a blend of individually entrapped glucoamylase and pullulanase in calcium alginate beads. They found that the binding of glucoamylase and pullulanase to alginate, before formation of calcium alginate beads, ensured the entrapment of these individual enzymes and also contributed to enhanced thermostabilization. Teotia et al. (2001) and Roy and Gupta (2002) also showed that both glucoamylase and pullulanase were bound to alginate.

Immobilization of enzymes exhibits many advantages such as reusability of the catalyst and improved enzyme stability under process conditions. Immobilization also allows the use of enzymes in various reactor designs like packed or fluidized beds. Thus, lower capital/energy costs and better logistics are associated with a process using an immobilized system (Roy and Gupta, 2004). Hence, the use of immobilized biocatalysts for starch hydrolysis in pullulan production offered the advantages of reusing the enzyme molecules, improved enzyme stability and using the enzyme molecules in repeated batch reactions when necessary.

3.2. Effect of initial substrate concentration on pullulan production

Hydrolysed potato starch waste was diluted to contain 30, 50, 70 and 90 g/l glucose and supplemented with 0.6 g/l $(NH_4)_2SO_4$, 0.4 g/l yeast extract, 5.0 g/l K_2HPO_4, 0.2 g/l $MgSO_4{\cdot}7H_2O$ and 1.0 g/l NaCl and fermented with *A. pullulans*. Fermentation was performed in shake flasks at 28 °C and pH 7.5. The kinetics of pullulan, polysaccharide and biomass production together with sugar consumption for different initial sugar concentrations are given in Fig. 1. As seen in Fig. 1, polysaccharide concentration increased significantly with the increase of initial sugar concentration from 30 to 70 g/l but decreased slightly above the initial sugar concentration of 70 g/l. The highest concentration of polysaccharide (20.62 ± 0.62 g/l) was obtained with an initial sugar concentration of 70 g/l after 96 h incubation. The maximum concentrations of polysaccharide obtained with initial sugar concentrations of 30, 50 and 90 g/l were 12.73 ± 0.22 g/l, 16.21 ± 0.21 g/l and 18.7 ± 0.41 g/l, respectively.

Since *A. pullulans* produces various polysaccharides together with pullulan, the pullulan content of the polysaccharide produced by *A. pullulans* was determined. The maximum pullulan concentration (16.02 ± 0.41 g/l) was obtained at the 96th h of fermentation in the culture grown at the initial sugar concentration of 70 g/l. The maximum concentrations of pullulan obtained with initial sugar concentrations of 30, 50 and 90 g/l were 5.88 ± 0.22 g/l, 11.50 ± 0.21 g/l and 14.87 ± 0.41 g/l, respectively. The maximum pullulan productivity values were 0.094, 0.137, 0.167 and 0.155 g/l/h for initial sugar concentrations of 30, 50, 70 and 90 g/l, respectively. The pullulan proportions at the 96th h of fermentation were 46.2%, 70.9%, 77.7% and 79.5% of the total polysaccharide in the medium containing 30, 50, 70 and 90 g/l initial sugar, respectively. Our previous study (Ürküt et al., 2007) on the optimization of pullulan production by *A. pullulans* in a stirred tank bioreactor using a synthetic fermentation medium containing sucrose showed that almost 30% of the polysaccharide in the fermentation broth were other polysaccharides. Roukas and Biliaderis (1995) found that 70% of the total polysaccharide produced was pullulan when carob pod extract was used as the substrate. Israilides et al. (1994) used different substrates for pullulan production and found that the defined medium produced highly pure pullulan while the complex substrates contained a mixture of other polysaccharides. Roukas and Liakopoulou-Kyriakides (1999) found that 35% of crude polysaccharide produced from molasses was pullulan and they stated that the pullulan contents of the crude polysaccharide depend on the strain of the microorganism used and the chemical composition of the substrate. The results of this study showed that almost 20–30% of the polysaccharides in

the fermentation broth containing 50–90 g/l of initial sugar were other polysaccharides produced by *A. pullulans*. The lower content of pullulan (46.2%) in the fermentation medium containing 30 g/l initial sugar may be attributed to the shift of the metabolism of the organism at low substrate concentrations to produce polysaccharides other than pullulan. Hence it was concluded that the use of hydrolysed starch waste as substrate does not have an adverse effect on the pullulan content of the polysaccharide.

Polysaccharide and pullulan concentrations increased significantly with increase of initial sugar concentration from 30 to 70 g/l. Above 70 g/l sugar concentration, there was a decrease in pullulan and polysaccharide concentrations due to inhibition produced by the high sugar concentration, which is a characteristic of the batch culture. In relevant work from author's laboratory (Göksungur, Dağbağlı, et al., 2005), maximum pullulan concentration was obtained at 50 g/l initial sugar concentration in a stirred tank bioreactor using the same organism. There is a similar trend in other studies using the same organism. Shin, Kim, Lee, Kim, and Byun (1987) found that pullulan production was inhibited at high initial sucrose concentrations and stated that the yields could be increased by using fed-batch fermentation. Roukas (1998) studied the production of pullulan from pretreated beet molasses and observed the highest concentration of polysaccharide at an initial sugar concentration of 70 g/l. Lazaridou, Biliaderis, Roukas, and Izydorczyk (2002) and Lazaridou, Roukas, Biliaderis, and Vaikousi (2002) studied pullulan production from beet molasses and found that the pullulan concentration decreased with the increase of initial sugar concentration from 50 to 100 g/l. They stated that the decline in pullulan production encountered with high sugar concentrations in the medium was probably due to osmotic effects and a lower level of water activity. It is noteworthy that growing *A. pullulans* in hydrolysed starch did not change the pullulan production behaviour of the organism at different initial sugar concentrations.

Kinetics of pullulan and polysaccharide productions showed that maximum pullulan and polysaccharide concentrations were obtained at the 96th h of fermentation and decreased significantly afterwards for all the initial sugar concentrations (Fig. 1). This decrease in pullulan concentration could be due to the hydrolysis of pullulan by an endogenous glucoamylase (named glucoamylase B) that is released by the microorganism at later stages of the fermentation; this enzyme is capable of degrading pullulan (Lazaridou, Roukas, et al., 2002).

Biomass yields of 6.47 ± 0.21, 7.22 ± 0.19, 7.20 ± 0.25 and 7.01 ± 0.28 g/l were obtained from the fermentation medium containing 30, 50, 70 and 90 g/l initial sugar, respectively. The pH of the fermentation medium decreased during the first 72 h of fermentation from an initial value of 7.5 to 3.7. The increase in acidity of the fermentation broth is attributed to the synthesis of organic acids.

During fermentation, the concentration of residual sugars declined, following an inverse trend to those of biomass, polysaccharide and pullulan concentrations (Fig. 1). After 120 h of fermentation, almost complete sugar depletion was observed in culture media containing 30 and 50 g/l of initial sugar. At higher initial sugar concentrations, residual sugar concentration increased slightly. The residual sugar concentrations in the culture medium containing 70 and 90 g/l were 10.53 ± 0.11 and 16.3 ± 0.17 g/l, respectively.

There are studies in the literature reporting that the dextrose equivalent of the starch syrup and the composition of the starch hydrolysate are important factors for pullulan production. Wu et al. (2009) studied pullulan production from hydrolysed sweet potato by *A. pullulans* AP329 and found that hydrolysis with α-amylase alone and prolonged hydrolysis with amyloglucosidase resulted in lower yields of pullulan. They produced pullulan at higher yields by continuous hydrolysis of sweet potato with the pullulanase and the β-amylase in sweet potato itself. They achieved maximum yield

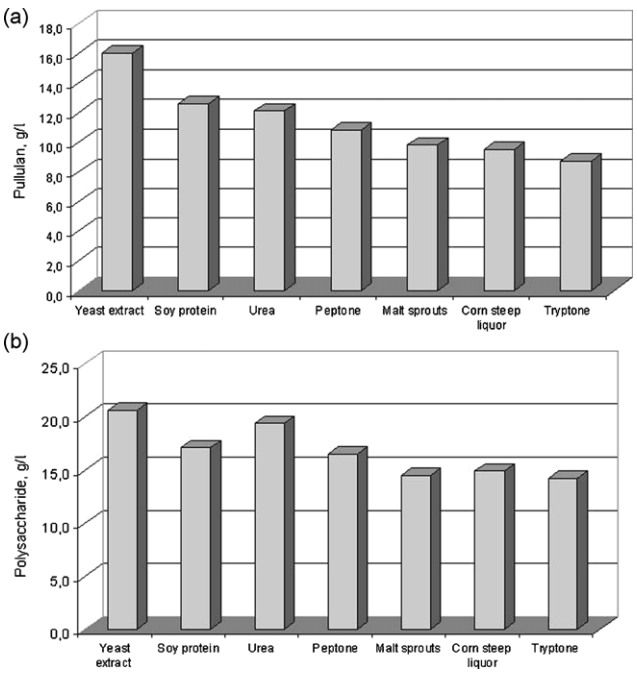

Fig. 2. The effect of different nitrogen sources on (a) pullulan and (b) polysaccharide production (28 °C, pH 7.5, the standard deviation of each experimental point ranged from 1.8 to 3.3).

(29.43 g/l) of pullulan at DE 45 and pH 5.5 for 96 h. Barnett et al. (1999) studied pullulan production from potato starch waste by *A. pullulans* and found that continued hydrolysis with pullulanase and amyloglucosidase gave higher yields with a better proportion of pullulan. However, prolonged hydrolysis did not improve the yield further. Our study optimized pullulan production process using potato starch waste, hydrolysed (DE 95.9) by Ca-alginate immobilized pullulanase and amyloglucosidase enzymes and considers fermentation parameters of initial sugar concentration, initial pH and incubation period for optimization of the process by response surface methodology.

3.3. Effect of nitrogen sources on pullulan production

Yeast extract is a commonly used nitrogen source in pullulan production and other bioprocesses. However, the high cost of yeast extract has a negative impact on the economics of its use in industrial scale processes. To improve the economic parameters of pullulan fermentation, comparable nitrogen sources were substituted with yeast extract (0.4 g/l) on equivalent nitrogen bases using hydrolysed starch medium containing 70 g/l initial sugar concentration. The amount of each nitrogen source was calculated to give the equivalent nitrogen of 0.4 g/l yeast extract. The nitrogen sources and the amounts used (g/l) were: malt sprouts, 0.74; corn steep liquor, 0.609; soy protein, 0.436; peptone, 0.28; urea, 0.0897; tryptone, 0.309. The results showed that pullulan and polysaccharide productions were markedly influenced by the nitrogen source used. As seen in Fig. 2a, yeast extract yielded the highest level of pullulan when compared with other nitrogen sources (16.02 ± 0.41 g/l). Soy protein and urea yielded 12.60 ± 0.24 g/l and 12.09 ± 0.18 g/l of pullulan, respectively. The other nitrogen sources were utilized by the microorganism, however they yielded lower pullulan levels than yeast extract (Fig. 1a). As seen in Fig. 2b, yeast extract and urea yielded similar polysaccharide levels of 20.62 ± 0.62 g/l and 19.38 ± 0.48 g/l, respectively while the other nitrogen sources yielded lower concentrations of polysaccharide. Our results showed that none of the nitrogen sources gave pul-

Table 4
ANOVA for pullulan concentration (R^2 = 0.995).

Source	DF[a]	Seq SS[b]	Adj SS[c]	Adj MS[d]	*F*	*P*
Regression	9	167.228	167.2277	18.5809	217.13	<0.001
Linear	3	64.332	64.3324	21.4441	250.59	<0.001
Square	3	98.623	98.6233	32.8744	384.17	<0.001
Interaction	3	4.272	4.2721	1.4240	16.64	<0.001
Residual error	10	0.856	0.8557	0.0856		
Lack of fit	5	0.606	0.6065	0.1213	2.43	0.176
Pure error	5	0.249	0.2493	0.0499		
Total	19	168.083				

[a] Degrees of freedom.
[b] Sequential sum of squares.
[c] Adjusted sum of squares.
[d] Adjusted mean square.

lulan concentrations as high as that obtained with yeast extract. Although urea gave polysaccharide concentration similar to yeast extract, pullulan content of the polysaccharide obtained with urea was lower (62.4%) than that of yeast extract (77.7%). Nitrogen availability was found to be an important factor for pullulan biosynthesis and the effect of nitrogen source upon pullulan synthesis by *A. pullulans* has been previously studied (Auer & Seviour, 1990; Lazaridou, Biliaderis, et al., 2002; Reed-Hamer & West, 1994; Schuster, Wenzig, Mershmann, 1993; Seviour, Stasinopoulos,Auer & Gibbs, 1992; Thirumavalavan et al., 2009). Similar to the findings of this study, Thirumavalavan et al. (2009) produced pullulan from coconut water and coconut milk by *A. pullulans* and found the highest pullulan concentrations when yeast extract was used as the nitrogen source followed by $NaNO_3$ and $(NH_4)_2SO_4$. Reed-Hamer and West (1994) studied the effect of some complex nitrogen sources including tryptone, peptone, soytone, casamino acids and corn steep liquor on pullulan production by *A. pullulans* in a medium where sucrose or corn syrup served as the carbon source. They found that the growth of the microorganism on soytone resulted in the highest level of pullulan production. Auer and Seviour (1990) found that pullulan production was highest when NH_4NO_3 and $(NH_4)_2SO_4$ served as the nitrogen source.

Further investigation was carried out for adjusting the optimum level of yeast extract and it was found that 0.4 g/l yeast extract concentration was optimum for pullulan production by *A. pullulans*. At higher nitrogen level, pullulan concentration decreased (data not shown). It is generally accepted that pullulan is produced under nitrogen limiting conditions and that high levels of nitrogen in the medium repress exopolysaccharide formation and instead encourage production of biomass (Seviour et al., 1992). Schuster et al. (1993) studied pullulan production from sucrose containing synthetic medium in batch and continuous modes of fermentation. They found that the formation rate of pullulan increased up to ammonium sulphate concentrations of 1.2 g/l, however at higher nitrogen levels, the product formation rate decreased since the microorganism was able to grow more intensively. Lazaridou, Biliaderis, et al. (2002) and Lazaridou, Roukas, et al. (2002) studied production of pullulan from molasses and found that pullulan formation occurs only after NH_4^+ is exhausted. They suggested that NH_4^+ may regulate the activities of key enzymes, causing a shift in carbon flow to biomass production at the expense of polysaccharide synthesis when its concentration in the medium exceeds a certain level.

3.4. Optimization of pullulan production using response surface methodology

Our preliminary experiments showed that the incubation time, initial substrate concentration and initial pH influenced the production of pullulan by *A. pullulans* P56. Thus response surface methodology was used to determine the optimum levels of these parameters leading to a maximum pullulan synthesis. The levels of these factors (Table 2) used in the optimization studies by RSM were determined by preliminary experiments. The effect of the three previously mentioned variables, each at three levels and their interactions on pullulan production has been determined by carrying out 20 experiments given by the face centered design (Table 3).

Analysis of variance (ANOVA) for the concentration of pullulan is presented in Table 4. The analysis gives the value of the model and determines the requirement of a more complex model with a better fit. As shown in Table 4, R^2 was 0.990 indicating that the model as fitted explained 99.0% of the variability in pullulan concentration. *F*-test for regression was significant at a level of 5% ($P < 0.05$) indicating that the model is fit and can adequately explain the variation observed in pullulan concentration with the designed levels of the factors. If the *F*-test for lack of fit is significant, then a more complicated model is needed to fit the data. As seen in Table 4, the lack of fit (0.176) was not significant at the 5% level ($P > 0.05$) indicating that the experimental data obtained fitted well with the model. These results show that the model chosen can satisfactorily explain the effects of incubation time, initial substrate concentration and initial pH on pullulan production by *A. pullulans* P56 in shake flask cultures using hydrolysed potato starch as substrate.

Twenty experiments were carried out from the design and by applying multiple regression analysis on the experimental data, the following second order polynomial equation was found to explain pullulan production by *A. pullulans*.

$$Y = -88.0150 + 0.344113X_1 + 0.820343X_2 + 15.3773X_3 - 0.00232094X_1^2 - 0.00612091X_2^2 - 1.35836X_3^2 + 0.0240990X_1X_3 + 0.0208813X_2X_3 \quad (2)$$

where X_1, X_2 and X_3 are the actual levels of factors shown in Table 2.

Regression analysis (Table 5) of the experimental data showed that initial pH, agitation speed and incubation time had positive linear effects on pullulan production ($P < 0.05$). Probability (*P*) values were used as a tool to check the significance of each of the coefficients. The smaller the magnitude of *P* value, the more significant was the correlation with the corresponding coefficient. Among the three factors tested, initial pH had the highest impact on pullulan concentration as given by the highest linear coefficient (15.3773), followed by initial substrate concentration (0.820343) and incubation time (0.344113). These factors also showed significant negative quadratic effects on pullulan production indicating that pullulan concentration increased as the level of these factors increased and decreased as the level of these parameters increased above certain values. Interaction between these parameters was also significant. The interactions between incubation time–initial pH, initial substrate concentration–initial pH were significant as shown by low *P* values ($P < 0.05$) for interactive terms. But the interaction between

Table 5
Estimated regression coefficients for pullulan concentration.

Term	Coefficient	SE coefficient	*T*	*P*
Constant	−88.0150	9.41296	−9.350	<0.001
Time	0.344113	0.06888	4.996	<0.001
Sugar conc.	0.820343	0.07593	10.804	<0.001
pH	15.3773	2.70411	5.687	<0.001
Time × time	−0.00232094	0.00031	−7.579	<0.001
Sugar conc. × sugar conc.	−0.00612091	0.00044	−13.879	<0.001
pH × pH	−1.35836	0.17640	−7.700	<0.001
Time × sugar conc.	−0.000329948	0.00022	−1.531	0.157
Time × pH	0.0240990	0.00431	5.592	<0.001
Sugar conc. × pH	0.0208813	0.00517	4.038	0.002

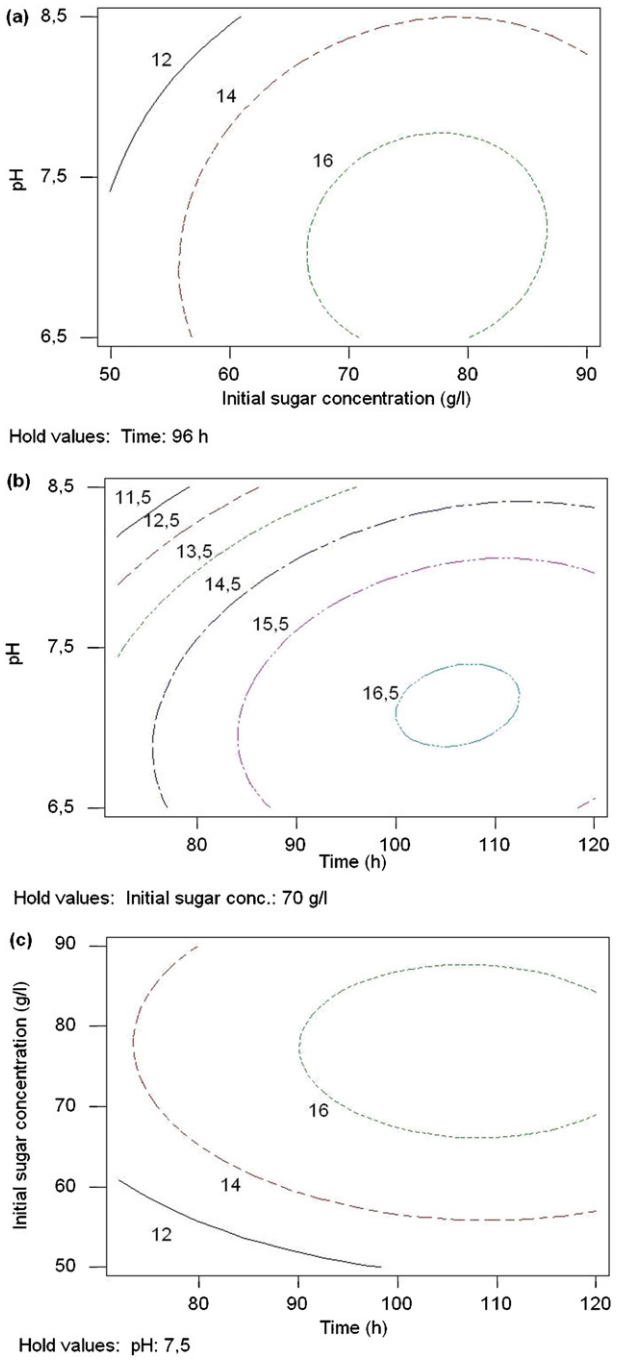

Fig. 3. Contour plots for pullulan concentration at varying concentrations of (a) initial sugar concentration and initial pH (b) time and initial pH (c) time and initial sugar concentration at a constant middle level of the other factor.

incubation time–initial substrate concentration was found to be insignificant as given by *P* value above 0.05. Hence this term was excluded from the polynomial Eq. (2) used for this model.

Fig. 3a–c shows the contour plots of pullulan concentration for each pair of factors by keeping the other factor constant at its middle level. Maximum pullulan production was obtained at middle level of each pair of factors at a constant middle level of the other factor. Further increase in these factors above the middle level (incubation time: 110 h, initial substrate concentration: 80 g/l, initial pH: 7.3) showed decrease in pullulan concentration. In order to determine the maximum pullulan concentration corresponding to the optimum levels of incubation time, initial substrate concentration and initial pH, a second order polynomial model was used to calculate the values of these variables. The fitting of the experimental data to Eq. (2) allowed the determination of the levels of incubation time (X_1 = 111.84 h), initial substrate concentration (X_2 = 79.4 g/l) and initial pH (X_3 = 7.26) giving a maximum pullulan concentration of 19.63 g/l. The above data optimizes pullulan production from hydrolysed potato starch waste by *A. pullulans* P56 in shake flask culture.

The final fermentation was performed using hydrolysed starch medium with the optimized levels of incubation time (111.84 h), initial substrate concentration (79.4 g/l) and initial pH (7.26) given by the model. Maximum pullulan production (19.2 ± 0.42 g/l) which was slightly lower than the value given by the model (19.63 g/l), was obtained at 111.84 h of fermentation. Compared to the maximum pullulan concentration (16.02 ± 0.41 g/l) obtained before the optimization studies, optimization of the process led to a 20% increase in pullulan production.

4. Conclusion

Response surface methodology, including an experimental design, regression analysis and model generation was an effective method for the optimization of pullulan production from potato starch waste hydrolysate. RSM was used to determine the effects of three important factors (incubation time, initial substrate concentration and initial pH) on pullulan production by *A. pullulans*. The model generated by RSM in this study satisfied all the necessary arguments for its use in the optimization process. By fitting the experimental data to a second order polynomial equation, the optimum levels of above mentioned variables were determined. Using the optimum levels of fermentation parameters, a maximum pullulan concentration of 19.2 ± 0.42 g/l was obtained. Compared to the pullulan concentration obtained before the optimization studies, pullulan production was increased by 20% in the optimized conditions. Thus, potato starch waste could be a potential substrate for developing an economical production medium for pullulan fermentation. This is the first report on the use of response surface methodology to optimize pullulan production from potato starch waste substrate. Future studies for increasing the pullulan concentration will focus on pullulan production, using starch waste

hydrolysates rich in maltose (hydrolysed by β-amylase) and the optimization of the dextrose equivalent values of the hydrolysed starch waste.

Acknowledgements

This research was supported by Ege University Scientific Research Project Fund through Project No: 2009 MÜH 035. We also thank Mrs. Huriye Göksungur for checking the language of the manuscript.

References

AOAC. (1999). In P. Cunniff (Ed.), *Official methods of analysis of the association of official analytical chemist*. Washington, DC: AOAC, pp. 1-2-12-16.

Auer, D. P. F., & Seviour, R. J. (1990). Influence of varying nitrogen sources on polysaccharide production by *Aureobasidium pullulans* in batch culture. *Applied Microbiology and Biotechnology*, *32*, 637–644.

Barnett, C., Smith, A., Scanlon, B., & Israilides, C. J. (1999). Pullulan production by *Aureobasidium pullulans* growing on hydrolysed potato starch waste. *Carbohydrate Polymers*, *38*, 203–209.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.

Göksungur, Y., Dağbağlı, S., Uçan, A., & Güvenç, U. (2005). Optimization of pullulan production from synthetic medium by *Aureobasidium pullulans* in a stirred tank reactor by response surface methodology. *Journal Chemical Technology and Biotechnology*, *80*, 819–827.

Göksungur, Y., Gündüz, M., & Harsa, S. (2005). Optimization of lactic acid production from whey by L casei NRRL B-441 immobilized in chitosan stabilized Ca-alginate beads. *Journal of Chemical Technology and Biotechnology*, *80*, 1282–1290.

Israilides, C., Scanlon, B., Smith, A., Harding, S. E., & Jumel, K. (1994). Characterization of pullulans produced from agro-industrial wastes. *Carbohydrate Polymers*, *25*, 203–209.

Jiang, L. F. (2010). Optimization of fermentation conditions for pullulan production by *Aureobasidium pullulans* using response surface methodology. *Carbohydrate Polymers*, *79*, 414–417.

Khatoon, S., Sreerama, Y. N., Raghavendra, D., Bhattacharya, S., & Bhat, K. K. (2009). Properties of enzyme modified corn, rice and tapioca starches. *Food Research International*, *42*, 1426–1433.

Lazaridou, A., Biliaderis, C. G., Roukas, T., & Izydorczyk, M. (2002). Production and characterization of pullulan from beet molasses using a nonpigmented strain of *Aureobasidium pullulans* in batch culture. *Applied Biochemistry And Biotechnology*, *97*, 1–22.

Lazaridou, A., Roukas, T., Billiaderis, C. G., & Vaikousi, H. (2002). Characterization of pullulan produced from beet molasses by *Aureobasidium pullulans* in stirred tank reactor under varying agitation. *Enzyme and Microbial Technology*, *31*, 122–132.

Leathers, T. D., Nofsinger, G. W., Kurtzman, C. G., & Bothast, R. J. (1998). Pullulan production by color variant strains of *Aureobasidium pullulans*. *Journal of Industrial Microbiology*, *3*, 231–239.

Li, C., Bai, J., Cai, Z., & Ouyang, F. (2002). Optimization of a cultural medium for bacteriocin production by *Lactotococcus lactis* using response surface methodology. *Journal of Biotechnology*, *93*, 27–34.

Lin, Y., Zhang, Z. S., & Thibault, J. (2007). *Aureobasidium pullulans* batch cultivations based on a factorial design for improving the production and molecular weight of exopolysaccharides. *Process Biochemistry*, *42*, 820–827.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, *31*, 426–428.

Myers, R. H., & Montogomery, D. D. (1995). *Response surface methodology: Process and product optimization using designed experiments*. New York: John Wiley & Sons, Inc.

Reed-Hamer, B., & West, T. P. (1994). Effect of complex nitrogen-sources on pullulan production relative to carbon source. *Microbios*, *80*, 83–90.

Roukas, T. (1998). Pretreatment of beet molasses to increase pullulan production. *Process Biochemistry*, *33*, 805–810.

Roukas, T. (1999a). Pullulan production from deproteinized whey by *Aureobasidium pullulans*. *Journal of Industrial Microbiology and Biotechnology*, *22*, 617–621.

Roukas, T. (1999b). Pullulan production from brewery wastes by *Aureobasidium pullulans*. *World Journal of Industrial Microbiology and Biotechnology*, *15*, 447–450.

Roukas, T., & Biliaderis, C. G. (1995). Evaluation of carob pod as a substrate for pullulan production by *Aureobasidium pullulans*. *Applied Biochemistry and Biotechnology*, *55*, 27–44.

Roukas, T., & Liakopoulou-Kyriakides, M. (1999). Production of pullulan from beet molasses by *Aureobasidium pullulans* in a stirred tank fermentor. *Journal of Food Engineering*, *40*, 89–94.

Roy, I., & Gupta, M. N. (2002). Purification of a bacterial pullulanase on a fluidized bed of calcium alginate beads. *Journal of Chromatography A*, *950*, 131–137.

Roy, I., & Gupta, M. N. (2004). Hydrolysis of starch by a mixture of glucoamylase and pullulanase entrapped individually in calcium alginate beads. *Enzyme and Microbial Technology*, *34*, 26–32.

Schuster, R., Wenzig, E., & Mersmann, A. (1993). Production of the fungal exopolysaccharide pullulan by batch-wise and continuous fermentation. *Applied Microbiology and Biotechnology*, *39*, 155–158.

Seviour, R. J., Stasinopoulos, S. J., Auer, D. P. F., & Gibss, P. A. (1992). Production of pullulan and other exopolysaccharides by filamentous fungi. *Critical Reviews in Biotechnology*, *12*, 279–298.

Shin, Y. C., Kim, Y. H., Lee, H. S., Cho, S. J., & Byun, S. M. (1989). Production of exopolysaccharide pullulan from inulin by a mixed culture of *Aureobasidium pullulans* and *Kluyveromyces fragilis*. *Biotechnology and Bioengineering.*, *33*, 129–133.

Shin, Y. C., Kim, Y. H., Lee, H. S., Kim, Y. N., & Byun, S. M. (1987). Production of pullulan from fed-batch fermentation. *Biotechnology Letters*, *9*, 621–624.

Singh, R. S., Singh, H., & Saini, G. K. (2009). Response surface optimization of the critical medium components for pullulan production by *Aureobasidium pullulans* FB-1. *Applied Biochemistry and Biotechnology*, *152*, 42–53.

Teotia, S., Lata, R., Khare, S. K., & Gupta, M. N. (2001). One-step purification of glucoamylase by affinity precipitation with alginate. *Journal of Molecular Recognition*, *14*, 295–299.

Thirumavalavan, K., Manikkadan, T. R., & Dhanasekar, R. (2009). Pullulan production from coconut by-products by *Aureobasidium pullulans*. *African Journal Of Biotechnology*, *8*, 254–258.

Ürküt, Z., Dağbağlı, S., & Göksungur, Y. (2007). Optimization of pullulan production using Ca-alginate-immobilized *Aureobasidium pullulans* by response surface methodology. *Journal of Chemical Technology and Biotechnology*, *82*, 837–846.

Vijayendra, S. V. N., Bansal, D., Prasad, M. S., & Nand, K. (2001). Jaggery: A novel substrate for pullulan production by *Aureobasidium pullulans* CFR-77. *Process Biochemistry*, *37*, 359–364.

Wu, S., Jin, Z., Tong, Q., & Chen, H. (2009). Sweet potato: A novel substrate for pullulan production by *Aureobasidium pullulans*. *Carbohydrate Polymers*, *76*, 645–649.