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Batch Fermentation Kinetics of Pullulan from *Aureobasidium pullulans* Using Low Cost Substrates

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Abstract: Batch pullulan fermentation kinetics of *Aureobasidium pullulans* from different low cost substrates was studied. In order to economize the process, different cheaper substrates such as cashew fruit juice, bakery waste, cassava flour and maize flour were attempted as a sole carbon source for the production of pullulan. A glucose based defined medium was used for comparison purposes. The higher yield of pullulan and increased uptake rate of substrate was noticed due to the rich content of sugar in cashew fruit juice. Almost three-fold increase in pullulan concentration (80 g L^{-1}) was achieved. The effects of initial pH and initial cashew fruit juice concentration had a strong effect on both the cell growth and pullulan production. Mathematical models which represent the batch pullulan fermentation kinetics were proposed. Simulations were made using the estimated kinetic parameter values and were compared with the experimental data.

Key words: Pullulan, *Aureobasidium pullulans*, cashew fruit juice, bakery waste, cassava flour, maize flour, Kinetic model

INTRODUCTION

Pullulan is an extra-cellular, linear, unbranched, water soluble, microbial polysaccharide, consisting of α-(1 → 6) linked maltotriose units (Leathers, 2002). A number of potential applications have been reported for this biopolymer as a result of its good film forming properties. Pullulan, can form thin films which are transparent, colorless, tasteless, odorless, tenacious, resistant to oil and grease and unaffected by small thermal variations. This polysaccharide is of economic importance with increased application in food, cosmetics, pharmaceutical, agricultural and chemical industries (Deshpande *et al*., 1992). For pullulan production, a variety of carbohydrate substrates has been employed in either chemically-defined (synthetic media) or non-defined media within the latter several agro-industrial wastes and by-products have been reported as potentially suitable substrates for pullulan synthesis. These include Grape skin pulp extract, starch waste, olive oil waste effluents and molasses (Israilides *et al*., 1994, 1998), olive oil wastes (Youssef *et al*., 1998), fuel ethanol byproducts (Leathers and Gupta, 1994), hydrolyzed potato starch waste (Barnett *et al*., 1999), glucosamine (Cescutti *et al*., 2002), beat molasses (Göksungur *et al*., 2004), brewery waste (Roukas, 1999a), deproteinized whey (Roukas, 1999b), carob pod (Roukas and Biliaderis, 1995), jaggery (Vijayendra *et al*., 2001). Although for some of these substrates the yields were similar to those obtained with conventional well-defined media, the structural features and purity of pullulan in the crude polysaccharide (alcohol-precipitated material) could vary widely depending on the carbon substrate, strain of the microorganism and fermentation conditions employed (Chi and Zhao, 2003). The effect of nitrogen source (Auer and Seviour, 1990; Roukas and Biliaderis, 1995; Seo *et al*., 2004), culture pH (Roukas and Biliaderis, 1995; Roukas, 1999b), incubation temperature (Chi and Zhao, 2003), dissolved oxygen levels (Gibbs and Seviour, 1996) as well as fermentor configuration (Gibbs and Seviour, 1992; Roukas and Mantzouridou, 2001), Minerals such as Zn^{+2}, Fe^{+3} (Reeslev and Jensen, 1995) were also reported. Many efforts have been devoted to optimize the fermentation conditions so as to improve the pullulan production, using the one factor at a time approach (Lee *et al*., 2001; Chi and Zhao, 2003). Improved pullulan productivity was reported by determining the optimal fermentation medium components and the initial pH using multivariable linear regression analysis (Tarabasz-Szymanska *et al*., 1999). A 2-level fractional factorial design was reported to reveal the effect of six physical, chemical and biological factors and their interactions on the fermentation of exopolysaccharides by A. pullulans were investigated by using response surface methodology (Lin *et al*., 2007).

The current trend in the utilization of carbon sources poses a great challenge in the commercial production of

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pullulan economically. In the present study, waste materials such as cashew apple juice and bakery effluent have been used as substrates. The effects of other starch rich agro-based products such as cassava flour and maize flour have also been studied. The cost of cassava flour and maize flour is approximately ten-fold lesser when compared to the cost of glucose (Rs. 320 kg^{-1}). Also the effect of initial pH, the effect of initial juice concentration and the effect of acid hydrolysis on various substrates were investigated. Kinetic modeling for representing the batch pullulan fermentation was reported.

MATERIALS AND METHODS

Micro organisms and growth conditions: *Aureobasidium pullulans* MTCC, 2195 used in this work was obtained from MTCC, Chandigarh. The organism was maintained on agar slants by keeping it in refrigerator at 4°C and was subcultured at fortnight time interval. The standard cultivation medium containing the following composition in g L^{-1}: Glucose, 30.0; K_2HPO_4, 5.0; KH_2PO_4, 3.0; KCl, 0.3; NaCl, 1.0; yeast extract, 0.2; $MgSO_4.7H_2O$, 0.2 and distilled water 1 L. The medium was autoclaved for 15 min at 121°C, cooled and the initial pH was adjusted to 7.0. A loop-full of culture from slant was transferred to 250 mL conical flask containing 50 mL culture medium. The flask was incubated at 30°C for 48 h in a rotary shaker incubator (Remi equipments, India) at 200 rpm. These cultures (5% v/v) were used to inoculate the production medium for all the fermentation studies.

Hydrolysis: Hydrolysis of different substrates was carried out using dilute sulfuric acid at 121°C (Choi and Mathews, 1996). Hydrolyzate of bakery waste, cassava flour and maize flour was prepared as follows: Ten gram of each sample along with 90 mL of different dilute sulfuric acid concentrations such as 1, 2, 3, 4 and 5% (v/v) were placed in an autoclave (Hitech equipment, India) and hydrolyzed for 1 h (121°C and 15 Psi). The sample was cooled in a tap water and filtered through filter paper. The filtrate was collected separately for all the runs and the sugar content was assayed as given below. The acid hydrolysates of all the substrates were neutralized using 1 N NaOH. Cashew fruit juice was used without hydrolysis for the fermentation studies.

Estimation of biomass and pullulan: At specific time intervals, the flasks were removed and the fermentation broth was analyzed for biomass and pullulan. Dry weight of total biomass (mycelia and yeast cells) was determined by centrifuging the fermentation broth (after appropriate dilution)in the high speed centrifuge (Indian equipment corporation, India) at 10000 x g for 20 min. The collected cell mass was washed twice with saline and distilled water and dried at 90°C till the mass reaches constant weight. The first supernatant was combined with the washing and the pullulan was precipitated using two volumes of ethanol at 4°C for 1 h. The precipitate obtained was washed with acetone and filtered through a pre-weighed Whatman No. 1 filter and dried at 90°C for constant weight (Vijayendra *et al*., 2001).

Estimation of total sugar: Total sugar concentration was measured by phenol-sulphuric acid method based on reaction with hot acidic medium (Krishnaveni *et al*., 1984). Glucose was dehydrated to hydroxyl methyl furfural and forms a green coloured product with phenol. Absorbance of the resulting solution was measured at 490 nm using Spectronic-20D Spectrophotometer (Elico India Pvt. Ltd).

RESULTS AND DISCUSSION

Effect of initial pH: To study the effect of initial pH on the growth of yeast and pullulan production, experiments were carried out by varying the initial pHs such as, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5. The relative concentrations of pullulan at pH 4.0 and 7.5 were 16.0 and 46.0 g L^{-1}, respectively, when compared to the pullulan concentration of 59.0 g L^{-1} at an optimum pH of 6.5. The enzyme activity may be maximum at the pH levels of 5.5 to 7.5. An optimal initial pH of 6.5 was reported for pullulan production from carob pod extract (Roukas and Biliaderis, 1995) and hydrolysed whey (Roukas, 1999b) in *Aureobasidium pullulans* batch fermentation. In acidic pHs say, from 4.0 to 6.0, the growth of an organism is much affected which reflects the pullulan synthesis. The generation time of the yeast was very high at the lower levels of pHs from 4.0 to 5.0. The cell mass and pullulan concentration obtained at lower pH levels was minimum when compared to higher pH levels. This is probably due to the influence of acidic pH on morphological character of the organism (Fig. 1).

Effect of acid concentration on the hydrolysis of substrates: In order to get an optimum conversion of different substrates, the experiments were carried out for various acid concentrations. Different concentration of sulfuric acid such as 1, 2, 3, 4 and 5% (v/v) were used to hydrolyse the substrates of bakery waste, cassava and maize. All hydrolysis studies were carried out in an autoclave for 1h at constant temperature (121°C). 2% (v/v) sulfuric acid concentration gave the highest glucose yield of 1.16, 0.8 and 0.77 g g^{-1} for the

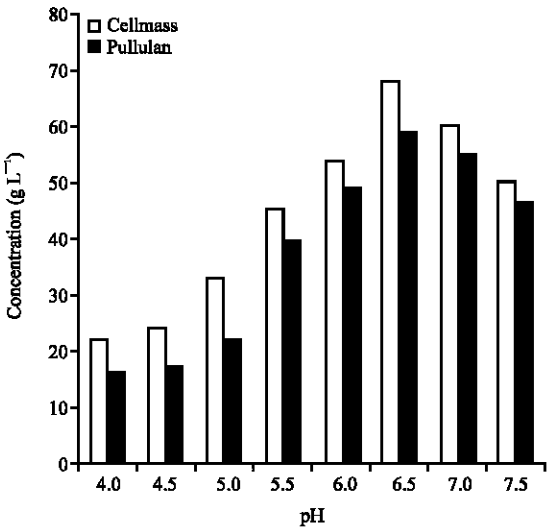

Fig. 1: Effect of initial pH on the growth of *A. pullulans* and synthesis of pullulan. Carbon source, 50 g L^{-1}, incubation temperature 28°C, inoculum size, 5% (w/v)

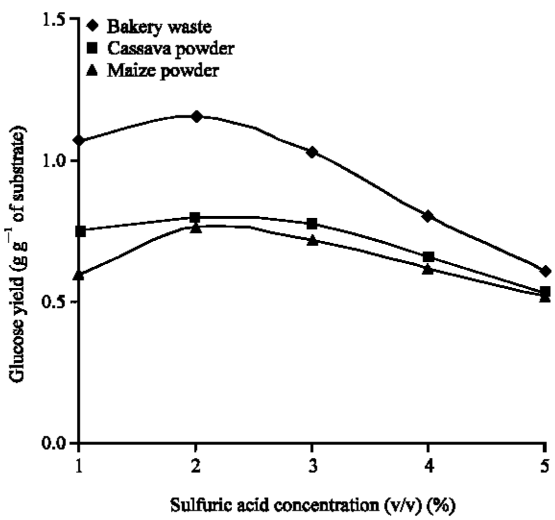

Fig. 2: Effect of acid concentration on the hydrolysis of different substrates

substrates of bakery waste, cassava powder and maize powder, respectively (Fig. 2). At higher sulfuric acid concentrations say 3, 4 and 5% (v/v), the yield dropped rapidly. The yield decreased as acid concentration increased. Higher glucose yield was obtained at a lower temperature, indicating that glucose decomposition rate was slow at the lower temperature(not shown in this text). Overall, the results of acid hydrolysis of different substrate indicate that the effect of acid concentration on the hydrolysis of substrates to glucose is more critical at higher concentrations due to the decomposition of monomeric sugars. For lower acid concentration levels,

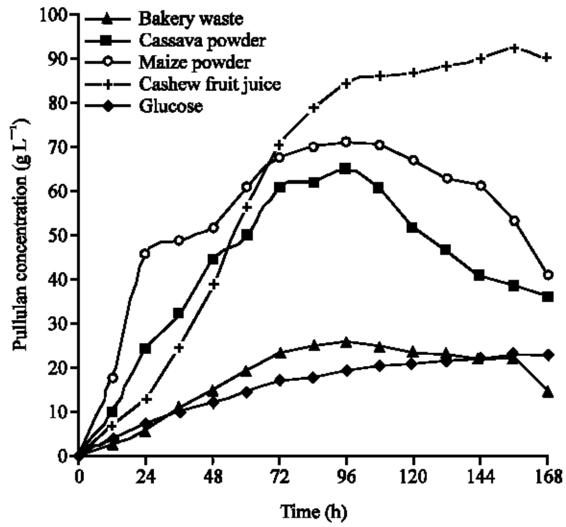

Fig. 3: Effect of different carbon source of *A. pullulans* fermentation. Carbon source, 50 g L^{-1}, initial pH, 6.5, incubation temperature 28°C, inoculum size, 5% (w/v)

increased conversions could be achieved by extending the hydrolysis time (Choi and Mathews, 1996). When compared to cassava and maize, a faster decomposition rate of monomers was observed for the case of bakery waste.

Effect of different substrates on pullulan fermentation: In order to economize the pullulan fermentation different agro-based wastes such as bakery waste, cashew fruit juice, cassava flour and maize flour were investigated as substrates. The fermentations were carried out for a period of 168 h for all the cases. The fermentation kinetics of pullulan production by *Aureobasidium pullulans* is represented in Fig. 3. The highest concentration of pullulan (92.5 g L^{-1}) was obtained at a fermentation period of 156 h for the case of cashew fruit juice, where as in glucose, the highest pullulan concentration obtained was 23 g L^{-1}. Pullulan content of 31.0 g L^{-1} was reported using synthetic medium after 120 h of fermentation in shake flasks with *A. pullulans* P 56 (Youssef *et al*., 1998). A uniform optimum time of 96 h was observed for all the other substrates. But, the highest pullulan concentration obtained for each substrates were different. The highest pullulan concentrations of 27, 65 and 71 g L^{-1} was obtained for bakery waste, cassava and maize, respectively. For the case of cassava and maize, the decreasing trend of pullulan concentration was obtained after the 96 h of fermentation. This shows that, the faster rate of utilization of sugars was noticed for the case of cassava and maize substrates. Similar results were

Table 1: Kinetic model equations for biomass, pullulan and Cashew fruit juice

Model	Equation
Biomass	
Logistic	$X_t = X_0 e^{\mu_0 t}/1-\left(X_0/X_m\right)\left(1-e^{\mu_0 t}\right)$
Pullulan	
LLP mode	$P_t = P_0 + \alpha X_0 \left[\frac{e^{\mu_0 t}}{1-\left(X_0/X_m\right)\left(1-e^{\mu_0 t}\right)}\right] + \frac{\beta X_m}{\mu_0}\ln\left[1-\frac{X_0}{X_m}\left(1-e^{\mu_0 t}\right)\right]1$
Cashew fruit juice	
LMLP model	$S_t = S_o - \gamma\left\{\frac{X_o e^{\mu t}}{1-(X_o/X_m)(1-e^{\mu t})} - X_o\right\} - \eta\left(\frac{X_m}{\mu}\right)\ln\left[1-\frac{X_o}{X_m}\left(1-e^{\mu t}\right)\right]$

Table 2: Kinetic parameter values for *Aureobasidium pullulans* fermentation

	Mohammad *et al.* (1995) Initial sucrose concentration (%) (w/v)				
Parameters	2.5	5	10	20	Present study (Cashew fruit juice)
Biomass					
μ_0 (h^{-1})	0.035	0.042	0.025	0.023	0.07
X_0 (g L^{-1})	0.161	0.110	0.142	0.151	1.00
X_m (g L^{-1})	0.501	0.792	0.923	0.721	92.00
Pullulan					
μ_0 (h^{-1})	0.035	0.042	0.025	0.023	0.09
α (g product/g biomass)	4.75	7.69	8.89	7.14	0.90
β (g product/g biomass h)	0.0092	0.0100	0.0204	0.0660	0.001
Substrate					
μ_0 (h^{-1})	0.035	0.042	0.025	0.023	0.09
γ (g substrate/g biomass)	3.67	3.16	4.80	5.60	0.98
η (g substrate/g biomass h)	0.0008	0.009	0.0168	0.0064	0.001

obtained for other substrates also. There may be several reasons for such variability in the production of pullulan including the chemical composition of the substrate, fermentation system and the utilization capacity of the strain for different substrates etc (Tsujisaka and Mitsuhashi, 1993).

Effect of different initial concentration of cashew juice: In order to find an optimum initial cashew juice concentration to get maximum cell growth and higher pullulan concentration the fermentations were carried out at different initial cashew juice concentration by making dilutions such as 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% (v/v). All the fermentations were carried out in an incubator shaker for 7 days at 28°C. The highest pullulan concentration of 83 g L^{-1} was obtained at an initial cashew fruit juice concentration of 50% (v/v). At higher dilution levels (10-40%), the growth and pullulan synthesis was significantly affected. This may be due to the availability of insufficient sugar for the fungus. And also an equal sugar contribution for the growth and polysaccharide synthesis was observed. At lower dilutions (60-100%), the sugar contribution favors the growth of the fungus so that the pullulan synthesis was affected much. The declining pullulan concentration with the increase in juice concentration was observed (Fig. 4). Similar result was observed from earlier literatures, with synthetic medium (Youssef *et al*., 1999), Beet Molasses and Synthetic Medium (Göksungur *et al*., 2004), carob pod (Roukas and Biliaderis, 1995) and jaggery (Vijayendra *et al*., 2001).

Kinetic modeling: The growth and product formation models (Dhanasekar *et al*., 2003) given in Table 1 were used for representing the batch pullulan fermentation kinetics using *Aureobasidium pullulans* utilizing cashew fruit juice. In earlier studies, different kinetic parameter values were used for the range of initial substrate concentrations studied (Mohammed *et al*., 1995). In the present study, a generalized model has been proposed for representing the batch pullulan fermentation kinetics. The model parameter values were evaluated by computation using MATLAB programme. The kinetic parameter values given in Table 2 were then used to simulate the profiles of biomass and pullulan during the entire course of fermentation. Comparisons were made between the experimental data and theoretical predictions (Fig. 5) shows that the results were closely followed each other (cell mass R^2 = 0.91; pullulan R^2 = 0.87; glucose R^2 = 0.85). The logistic model appears to provide adequate representation of the growth kinetics of *Aureobasidium pullulans*. Logistic incorporated Leudeking-Piret model (LLP) and the Logistic incorporated modified Leudeking-Piret model (LMLP)

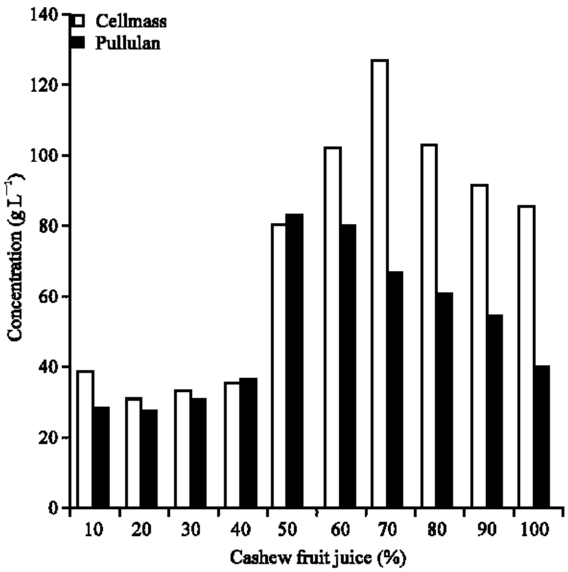

Fig. 4: Effect of cashew fruit juice concentration on *A. pullulans* growth and pullulan production. Initial pH, 6.5; incubation temperature, 28°C; inoculum size, 5% (v/v)

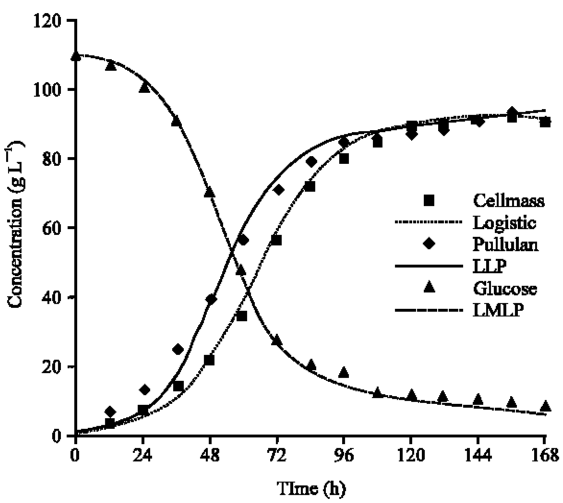

Fig. 5: Comparison of the experimental data (dots) for pullulan production using cashew fruit juice as substrate and simulations from Logistic (------), Logistic incorporated Leudeking-Piret model (——) and the Logistic incorporated modified Leudeking-Piret model (– – – – – –)

were used to predict the pullulan formation and substrate utilization kinetics, respectively. Only one response curve was shown in Fig. 5, but the models have been checked for all the studied substrate concentrations during the entire course of fermentation. Comparison between the experimental data and the theoretical prediction shows that, the logistic, logistic incorporated Leudeking-Piret and logistic incorporated modified Leudeking-Piret models for extra cellular polysaccharide *Aureobasidium pullulans* fermentation are close to reality.

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