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Full Length Research Paper

A novel fermentor system optimized for continuous production of pullulan

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A novel fermentor system containing *Aureobasidium pullulans* in polyurethane foam was designed to achieve continuous production of pullulan. *A. pullulans* cells were immobilized in 10 to 20 g polyurethane foam with pore size of 1000 Å. The system has specialized aeration provision with 80 perforations of 4 mm. Pullulan production achieved was approximately 37g/l in 18 cycles at 42°C, pH 5.5 and at aeration rate of 0.5 vvm. The concentration of sucrose and ammonium sulphate was 3 and 0.5%, respectively. This novel design could serve as an excellent fermentor system in industries for large scale continuous production of pullulan.

Key words: *Aureobasidium pullulans,* polyurethane foam, pullulan, immobilized cells.

INTRODUCTION

Aureobasidium pullulans is a polymorphic fungus, having four morphological growth forms which are mainly yeast like, young blastospores, swollen blastospores and chlamydospores. Yeast phase is responsible for pullulan production (Campbell et al., 2004). Pullulan is a linear homopolymer composed of maltotriose subunits inter-connected with α-1, 6 glycosidic linkages. The regular alternation of α-1, 4 and α-1, 6 bonds results in two distinctive properties; the structural flexibility and enhanced solubility in water (Leathers, 2002). Due to these features, this polymer is being exploited in food, cosmetic, medical and pharmaceutical industries (Singh et al., 2008, Gaur et al., 2010).). Pullulan generates a highly viscous solution at a relatively low concentration. It can be used for oxygen-impermeable films, fibers, thickening or extending agents, adhesives, or encapsulating agents, etc. Never-theless, its applications are extensive and more than 300 patents for its applications have been reported so far (Singh et al., 2008, Chi et al., 2009, Gaur et al., 2010).

Numerous methods have been described for the production of pullulan, including simple batch (Thirumavalavan et al., 2009), fed batch (Shin et al., 1987), continuous (Schuster et al., 1993), stirred tank fermentor (Lazaridou et al., 2002) as well as immobilized cells of *A. pullulans* in laboratory flask cultures or in simple traditional fermentor (Shin et al., 2004; Urkut et al., 2007). Recently, Singh et al. (2011) developed a packed bed reactor for constant production of pullulan using covalent immobilized pullulanse. None of these methods alone are feasible for high pullulan production. This is mainly due to higher viscosity of pullulan solution which hinders the aeration and proper mixing of the nutrients, resulting to poor yeast phase of growth during fermentation. Hence, a fermentor with novel design which overcomes limitations imposed by the high viscosity of this microbial metabolite is needed to obtain continuous production of pullulan.

MATERIALS AND METHODS

The immobilization of *A. pullulans* cells of wild type was done in a self-designed multistage continuous fermentor having an immobilization unit. The polyurethane foam quality and quantity were also optimized into the fermentor. The optimal conditions for pullulan production by *A. pullulans* were 42°C, pH 5.5 in 48 h

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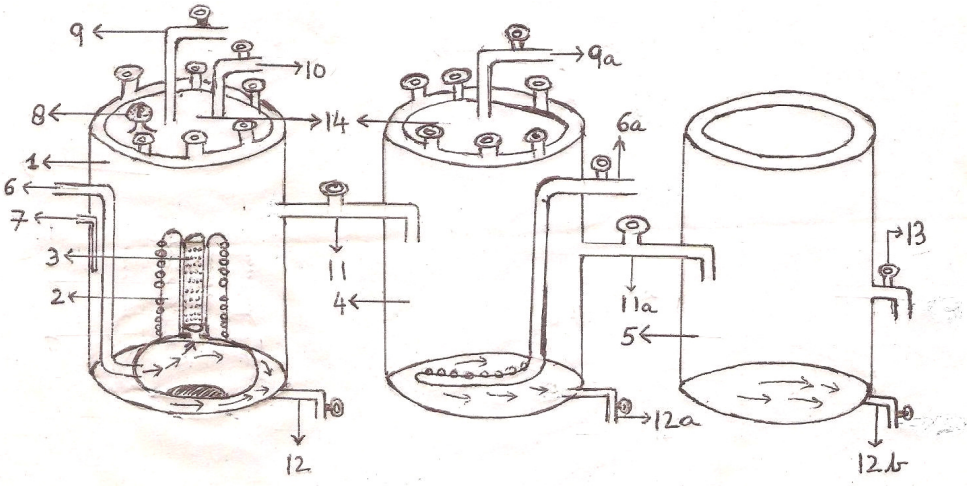

Figure 1. Schematic representation of multistage continuos fermentor (1, unit A ; 2, unit B ; 3, unit C ; 4, unit D ; 5, unit E ; 6, aeration unit ; 6a, aeration unit ; 7, thermostatic heating device ; 8, pressure gauge ; 9, exhaust ; 9a, exhaust ; 10, extra port for medium inlet ; 11, connecting pipe with regulator ; 11a, connecting pipe with regulator ; 12, outlet for dead yeast cells and washing ; 12a, outlet for dead yeast cells and washing; 13, drain pipe for pullulan outlet; 14, replaceable lid).

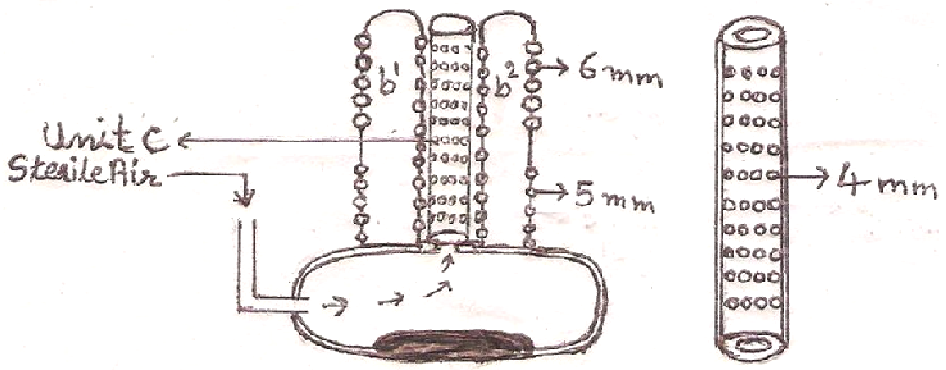

Figure 2. Unit B with b^1 and b^2 and unit C.

having 3% sucrose and 0.5% ammonium sulphate for production of pullulan (37 ± 1.0 g/l), therefore, similar parameters were considered for pullulan production through immobilized cells of *A. pullulans* on polyurethane foam in a self designed fermentor model with several specifications as described later. The grade of polyurethane foam was also optimized for better attachment of the cells for longer period. The grade is based on the pore size mainly classified as macropore, mesopore and micropore. Macropore (1000 Å) was selected for this study.

Design of the novel fermentor and its operational parameters

The fermentor was fabricated indigenously in the laboratory as per the design consisting basically of 5 units 'A', 'B', 'C', 'D' and 'E' (Figure 1). The unit A is of 10 L capacity with working volume of 7 L, fabricated in mild steel vessel. The main fermentor (unit 'A'), has a replaceable lid on the top having three ports, an exhaust for gas (CO_2), a pressure gauge and an extra port. Besides this, unit A comprises an aeration unit directly connected to the bottom of unit 'B'. Sterile air is supplied only to unit 'A' and 'B' as per the design in the fermentor. All the fermentation vessels have an outlet in the bottom for total decantation during washing or for any other purpose like, release of dead yeast sludge from the fermentor. A provision of thermostatic heating device was made in the unit 'A' to control the desirable temperature (42°C) for thermostable *A. pullulans* used in this investigation.

The unit 'B' is centrally fitted in the middle of the main fermentor (unit A) and has an extended b^1 and b^2 segments having perforations on the outer and inner walls having a removable socket as immobilization unit 'C' which exists in the middle of b^1 and b^2 segments. The inner walls of unit b^1 and b^2 segments are in close contact with the unit 'C'. The outer walls of unit b^1 and b^2 have 5 perforations of 5 mm in size on the lower and 5 perforations of 6 mm on the upper side (Figure 2). These pores help in the circulation of air from Unit 'B', 'C' and finally to 'A' continuously. The upper portion of the unit 'B' is dome shaped without perforation to reflect back the air into the lower segments of b^1 and b^2, helping the medium for proper mixing. The design of the flat upper portion of b^1 and b^2 (non-domed) was also compared with the dome shaped

structure. However, dome-shaped design was found to be better for yeast phase of growth (data not shown).

The unit 'C' is barrel shaped, 10 inches long having 50 mm radius, made up of mild steel, a removable unit, packed with polyurethane foam along with *A. pullulans* culture (50 x 10^6 CFU/g) for immobilization. Therefore, unit 'C' is the main immobilization unit. The top of the unit 'C' is opened for regular contact with the medium and also releases the air and respired CO_2 to avoid back pressure in it. The design of unit 'C' is especially made for polyurethane having macropores (1000 Å). Unit 'C' has 80 perforations of 4 mm each throughout the walls. These numbers and size were optimized for pullulan production, particularly for this novel model of fermentor, especially for unit 'C'. This design is presented in Figure 2.

The main fermentor unit 'A' is interconnected with unit 'D' and 'E' having 15 L capacity to achieve a multistage continuous fermentation system, and the flow of the medium from unit 'A' to 'D' and 'E' is through gravitational force as well as water level principles to avoid the back flow. The unit 'D' is closed on the top with an air exhaust outlet having an aeration unit with sparging of air in the bottom side, regulated to any capacity depending on the requirement. Both units 'D' and 'E' have a bottom outlet for decantation of sludge and washing materials.

The number of fermented cycle was calculated on the basis of the capacity of the fermentor, that is, 7 liters effluent of the fermented medium from unit 'D' was considered as one cycle. The results of the biomass and pullulan production having almost similar count of the yeast cells were considered as efficient process parameters for pullulan production under scale 1. The changes in the production and other parameters by 5 to 10% variation were considered as significant change in the production under scale 2, while the scale 3 meant poor production showing 10 to 15% loss in productivity. In this experiment, the immobilization efficiency was evaluated on the basis of no change in continuous production of pullulan, biomass and yeast-like cells in the main fermentor. The design of the basic unit of fermentor was totally standardized on the basis of biomass in the yeast phase essential for the pullulan production.

Support material

The polyurethane foam having micropores (less than 20 Å pore width), mesopores (between 20 Å - 500 Å widths) and macropores (more than 500 Å widths) as solid support material for immobilization of *A. pullulans* cells for unit 'C' were optimized for pullulan production. The macro pore polyurethane foam is designated as polyurethane grade B (1000 Å). Different grade of polyurethane foam was purchased from Sigma Chemicals, St. Louis, MO, U.S.A..

Inoculum preparation for immobilization of *A. pullulans* on the polyurethane foam

Cell suspension was prepared by inoculating 1 ml of 48 h grown culture in 200 ml nutrient broth and incubated at 42°C for 24 h to achieve active exponential phase of the culture having 50 x 10^6 cfu/ml in the yeast phase of cells soaked in 10 to 20 g of polyurethane foam already packed in the perforated column (unit 'C') for evaluation of the suitable quantity of solid matrix and, the packed column was placed in the socket of unit 'B'. This design was further evaluated with different aeration rate in this unit.

Calculation of percentage of the yeast cells in the fermentor

The percentage of yeast cells in the fermentor was estimated by hemocytometer. The four types of structures viz. yeast cells, blastospores, thin mycelium and chlamydospores were taken into account for total cells, out of which yeast cells present were calculated as percentage level. The mycelium of 100 µm size was considered as active phase for counting.

Optimal physico-chemical parameters for *A. pullulans* strain for pullulan production by immobilization process in a novel model of fermentor

A. pullulans strain used in this investigation was already optimized at different physico-chemical and nutritional levels in the flask shake culture as well as in non-stirred batch fermentation process which are as follows: optimum temperature 42°C, pH 5.5, aeration 0.5 vvm, sucrose concentration 3%, ammonium sulphate concentration 0.5%.

Effect of polyurethane foam quality and quantity in unit 'C' for pullulan, biomass production and yeast phase of growth

Different quality and quantity of polyurethane foam were evaluated for the pullulan, biomass and yeast phase of growth. For this, 10, 15 and 20 g polyurethane foam was packed in immobilization unit 'C'.

Effect of different aeration rate in the immobilization unit 'B' for pullulan and biomass production

Aeration rate plays a very important role in the production of pullulan and biomass, especially in this model. Therefore, different aeration rates viz., 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 vvm were evaluated for the production of pullulan, biomass and percentage of yeast-like cells in the unit 'B'.

Effect of numbers and size of pores in the unit 'C' on pullulan and biomass production

The number and size of pores in the unit 'C' have affected the pullulan and biomass production. Therefore, different sizes viz., 2 and 4 mm and numbers viz., 40, 60 and 80 of the pore were evaluated for pullulan and biomass production in unit 'C'.

Effect of number, size and location of pores in the unit 'B' on pullulan and biomass production

Effect of different pores sizes viz., 3, 4, 5, 6, 7 mm, numbers 3, 5, 7 and locations (upper and lower side) were optimized for optimum pullulan production on the outer walls of unit 'B' into the fermentor.

Effect of dilution rate of the nutrient medium on continuous production of pullulan, biomass and yeast cells

Effect of the nutrient dilution rate viz., 20, 40, 60, 80 and 100 on the continuous production of pullulan, biomass and yeast cells was critically evaluated.

Optimization of the immobilization capability of *A. pullulans* on polyurethane foam in different fermentation cycle

The number of fermentation cycles viz., 2, 4, 6, 8, 10, 12, 14, 16 and 18 were calculated on the basis of the capacity of the fermentor, that is, the release of 7 L effluent from unit D was considered as one cycle. The immobilization capability of *A.*

Table 1. Effect of polyurethane foam quantity and quality in unit 'C' for pullulan and biomass production.

Polyurethane foam quantity (g)	Polyurethane foam quality on basis of pore size (A°)	Pullulan production (g/l)	Biomass production (g/l)	Yeast like cell (%)
10	Micro-pore	15.5 ± 0.8	9.0 ± 1.0	25 ± 1.0
	Meso-pore	22.0 ± 0.5	14.2 ± 0.5	33 ± 0.8
	Macro-pore	28.5 ± 1.0	19.0 ± 1.0	45 ± 0.9
15	Micro-pore	26.5 ± 0.5	10.3 ± 0.8	49 ± 1.0
	Meso-pore	32.5 ± 1.0	15.4 ± 1.0	65 ± 0.9
	Macro-pore	37.2 ± 1.0	20.5 ± 0.9	80 ± 1.0
20	Micro-pore	26.0 ± 1.0	9.6 ± 1.0	47 ± 1.0
	Meso-pore	32.0 ± 0.5	14.7 ± 0.5	63 ± 0.9
	Micro-pore	36.0 ± 1.0	19.0 ± 1.0	78 ± 1.0

pullulans was optimized on the polyurethane foam in different cycles.

Estimation of Pullulan and Biomass

1. Isolation and purification of extracellular polysaccharide:

After fermentation, the culture medium was heated at 100°C in water bath for 15 minutes, cooled to room temperature and centrifuged at 12,000 rpm (9660g) at 4°C for 10 minutes to remove cells and other precipitates. Three ml of the supernatant were transferred into a test tube, and then 6 ml of cold ethanol (99% ethanol) was added to the test tube and mixed thoroughly and held at 4°C for 12 hours to precipitate the extracellular polysaccharide. After removal of the residual ethanol, the precipitate was dissolved in 3 ml of deionized water at 80° C and the solution was dialyzed against deionized water for 48h to remove small molecules in the solution. The exopolysaccharide was precipitated again by using 6 ml of the cold ethanol and the residual ethanol was removed, the precipitate was then dried at 80°C to a constant weight (Badr-Eldin et al., 1994). Pullulan weight was measured using electronic balance (Sartorius Limited, U.S.A.) and expressed in gram/liter.

2. Hydrolysis of the purified extracellular polysaccharide and assay of reducing sugar:

To assay the component of the extracellular polysaccharide, the purified precipitate was vacuum desiccated to no alcohol level by using a vacuum pump, then dissolved in 3 ml deionized water at 80°C water bath. The dissolved substrate was hydrolyzed by incubating the mixture of 0.5 ml of the substrate, 0.4 ml of 0.2 Mol Na_2HPO_4 -0.1 Mol acid citric buffer (pH 5.0) and 0.1 ml pullulanase (Sigma Chemicals, St. Louis, MO, USA) for 2 hour at 40°C (Su, 1986). The released reducing sugar was determined by using modified Dinitro Salicylic Acid (DNS) method (Lee *et al.,* 1999) for the confirmation of pullulan.

Statistical analysis

All the experiments were carried out in triplicate and the data were analysed at standard deviation level.

RESULTS

Fermentation kinetics of pullulan production, especially for biomass and yeast phase of growth were recorded at different time intervals in different designs of the fermentor along with immobilization unit with poly-urethane foam quality and quantity as support material for wild type of *A. pullulans* cells. The polyurethane foam having pore size of 1000 Å in 15 g with aeration rate of 0.5 vvm in the immobilization unit C having 50 x 10^6 cfu/g with 80 perforations of 4 mm was found to be the best in multistage continuous fermentation process for pullulan production. Further-more, unit B having b^1 and b^2 segments having 5 perforations of 5 mm size on the lower side and 5 per-forations of 6 mm size on the upper side with temperature 42°C, pH 5.5, sucrose concentration 3%, ammonium sulphate concentration 0.5% were found to be better for continuous production of pullulan (37 ± 1.0 g/l) in 18 cycles.

A. pullulans strain used in this investigation was already optimized at different physico-chemical and nutritional levels in non-stirred batch-fermentation system which is as follows: optimum temperature 42°C, pH 5.5, aeration 0.5 vvm, sucrose concentration 3%, ammonium sulphate concentration 0.5%. Pullulan production was 37 ± 1.0 g/l, while biomass and yeast-like cells were 21 ± 0.8 g/l and 80 ± 0.7 (%), respectively. The immobilization parameters along with the change in the configuration and design of the fermentor were also optimized in a multistage continuous design in order to achieve continuous production of pullulan.

Effect of polyurethane foam quality and quantity in unit 'C' for pullulan, biomass production and yeast phase of growth

Different quality and quantity of polyurethane foam were evaluated for pullulan, biomass and yeast phase of growth. For this, 10, 15 and 20 g polyurethane foam were packed in immobilization unit 'C'. It would be clear from the results that highest pullulan production was recorded

Table 2. Effect of different aeration rate in immobilization unit 'B' for pullulan and biomass production.

Aeration rate (vvm)	Pullulan production (g/l)	Biomass production (g/l)	Yeast like cell (%)
0.1	14.6 ± 1.0	07.1 ± 1.0	40 ± 1.0
0.2	19.0 ± 0.9	10.5 ± 0.9	52 ± 1.0
0.3	25.7 ± 1.0	14.6 ± 0.9	65 ± 0.9
0.4	31.8 ± 1.0	17.5 ± 0.8	72 ± 0.9
0.5	37.0 ± 1.0	20.4 ± 1.0	79 ± 0.8
0.6	36.4 ± 0.8	20.0 ± 0.9	79 ± 1.0
0.7	36.0 ± 1.0	19.0 ± 0.8	78 ± 1.0
0.8	35.1 ± 1.0	19.5 ± 1.0	77 ± 1.0
0.9	35.0 ± 1.0	19.0 ± 1.0	77 ± 0.9
1.0	34.5 ± 1.0	18.0 ± 1.0	76.5 ± 0.8

Table 3. Effect of the number and size of pores in unit 'C' on the production of pullulan, biomass and yeast like phase.

Pore size (mm)	Pore number	Pullulan production (g/l)	Biomass production (g/l)	Yeast like cell (%)
2	40	25.0 ± 1.0	10.1 ± 0.9	50 ± 1.0
	60	28.3 ± 0.9	13.9 ± 0.8	65 ± 0.9
	80	30.0 ± 1.0	17.5 ± 1.0	74 ± 1.0
4	40	32.5 ± 0.9	19.0 ± 1.0	77 ± 0.9
	60	36.2 ± 0.9	20.0 ± 0.9	78 ± 0.9
	80	37.1 ± 1.0	21.1 ± 1.0	79 ± 1.0

in macro-pores having pore size of 1000 Å in 15 g packing. Micro- and Meso-pores produced comparatively lesser pullulan, biomass and percentage of yeast cells (Table 1).

Effect of different aeration rate in the immobilization unit 'B' for pullulan and biomass production

Aeration rate plays a very important role in the production of pullulan and biomass especially in this model. Therefore, different aeration rates were evaluated for the production of pullulan, biomass and percentage yeast-like cells in the unit 'B' specially designed for aeration and holding unit 'C'. Highest pullulan production was reported at 0.5 vvm aeration rate which was optimal for pullulan production. Further increase in the aeration rate did not affect the pullulan production and the other parameters, while lesser aeration rate reduced the pullulan production (Table 2).

Effect of number and size of pores in the unit 'C' on pullulan and biomass production

The number and size of pores in the unit 'C' have affected the pullulan and biomass production. It is clear from the results indicated in Table 3 that maximum pullulan and biomass were produced in case of pore size of 4 mm in 80 numbers, while small size as well as lesser numbers of the pores reduced the pullulan production significantly. Hence, this particular size and number of the pores were used for unit 'C' in the novel fermentor model that produced higher pullulan for longer period without recharging of the immobilization unit with the active phase of the culture of *A. pullulans.*

Effect of numbers, size and location of pores in the unit 'B' on pullulan and biomass production

Effect of different pore size, numbers and location were optimized for optimum pullulan production on the outer walls of unit 'B' in the fermentor. Maximum pullulan and biomass production were reported in the pores size of 6 mm in 5 numbers located at the upper side walls and 5 mm in 5 numbers at the lower side walls of b^1 and b^2 of the main unit 'B' (Table 4). Further increase or decrease of pore size and numbers reduced pullulan production. Hence, this particular size and numbers were used in the design development of this novel fermentor in order to

Table 4. Effect of number, size and location of pores in unit B (b^1 and b^2).

Set of design	Pore location	Pore size (mm)	Pore number	Pullulan production (g/l)	Biomass production (g/l)	Yeast like cell (%)
1	Lower side	3	3	20.1 ± 1.0	09.0 ± 1.0	45 ± 1.0
		3	5	25.9 ± 1.0	12.3 ± 1.0	54 ± 0.9
	Upper side	4	3	28.3 ± 1.0	15.5 ± 0.8	63 ± 1.0
		4	5	32.0 ± 0.9	17.0 ± 0.9	69 ± 1.0
2	Lower side	5	3	35.0 ± 0.9	19.0 ± 0.9	74 ± 0.9
		5	5	36.5 ± 1.0	20.5 ± 0.9	79 ± 1.0
		7	7	32.5 ± 1.0	15.6± 1.0	70± 1.0
	Upper side	6	3	35.0 ± 1.0	19.0 ± 1.0	74 ± 1.0
		6	5	37.0 ± 1.0	21.0 ± 1.0	80 ± 0.9
		7	7	33.5 ± 1.0	16.5 ± 0.9	73.0 ± 1.0

Table 5. Effect of dilution rate of nutrient medium on continuous production of pullulan, biomass and yeast cells production.

Dilution rate (ml/h)	Pullulan production (g/l)	Biomass production (g/l)	Yeast like cell (%)
20	18.0 ± 1.0	12.4 ± 1.0	49 ± 1.0
40	25.5 ± 1.0	15.3 ± 0.9	60 ± 1.0
60	30.6 ± 0.9	17.6 ± 0.9	68 ± 0.9
80	36.2 ± 1.0	20.5 ± 1.0	79 ± 1.0
100	32.5 ± 1.0	18.5 ± 0.9	63 ± 0.9

achieve continuous production of pullulan.

Effect of dilution rate of the nutrient medium on continuous production of pullulan, biomass and yeast-like cells

Effects of the nutrient dilution rate on the continuous production of pullulan, biomass and yeast-like cells were critically evaluated. It is clear from the result in Table 5 that maximum pullulan, biomass and yeast-like cells were produced when the dilution rate was 80 ml/h, while below or above this value, the production reduced significantly. The viscosity was also measured through viscometer for correction in the factors (data not shown).

Optimization of fermentation cycle for pullulan production by immobilized cells of *A. pullulans*

The number of fermentation cycle was calculated on the basis of the capacity of the fermentor, that is, the release of 7 L effluent from unit D was considered as one cycle. The immobilization capability of *A. pullulans* was optimized on the polyurethane foam in different cycles. It is clear from the result that in 18 cycles, no change in the production of pullulan, biomass and yeast-like cells were reported (Table 6). Further cycle reduced the production of pullulan (Data not shown).

DISCUSSION

Pullulan is an important polysaccharide and has elastic, nontoxic, edible and oxygen impermeable transparent thin film forming capability just like polyethylene films; therefore, its commercial production for the preparation of biodegradable plastic has been started in Japan and USA (Singh et al., 2008 Gaur et al., 2010). The economical commercial pro-duction can only be achieved by continuous fermentation process which requires several scientific skills in designing a bioreactor either in simple continuous or using immobilization of *A. pullulans* cells on a suitable matrix. It is clear from the findings that multistage continuous fermentor is better than the single fermentor for this microbial metabolite. This strain of *A. pullulans* had already been optimized for pullulan production in the flask shake culture as well as non-stirred batch fermen-tation process. For immobilization, the poly-urethane foam of grade B having macropores (1000 Å) was used as solid matrix which could provide sufficient oxygen as well as microbial film forming capability in the immobilization unit 'C', and could

Table 6. Optimization of fermentation cycle for pullulan production by immobilized cells of *A. pullulans*.

Fermentation cycle	Pullulan production (g/l)	Biomass production (g/l)	Yeast like cell (%)
2	36.8 ± 1.0	20.0 ± 1.0	80 ± 1.0
4	37.2 ± 0.9	21.0 ± 1.0	80 ± 1.0
6	36.7 ± 0.8	21.2 ± 0.9	79 ± 1.0
8	36.9 ± 1.0	21.0 ± 0.9	80 ± 1.0
10	37.3 ± 0.9	20.9 ± 0.9	79 ± 0.9
12	37.0 ± 1.0	20.9 ± 0.8	80 ± 1.0
14	36.5 ± 0.9	21.4 ± 1.0	79 ± 1.0
16	36.6 ± 0.8	21.5 ± 1.0	80 ± 1.0
18	37.0 ± 0.9	21.4 ± 0.9	80 ± 0.9

Other fermentative parameters were same as in previous conditions.

continuously release *A. pullulans* cells in the yeast phase of the growth into the main fermentor (unit 'A'), where the cells grow in less viscosity with a constant biomass (chemostatic and turbostatic phase), and then passes in semi-fermented broth into the unit 'D' and in idophase into the unit 'E', simultaneously at the rate of 80 ml/h in a multistage continuous fermentor system in order to achieve constant pullulan content, resulting in higher productivity. An aeration system in the unit 'D' is always necessary because tropho- and partial idao-phase occur in this unit. Moreover, the total transformation of nutrients take place for the remaining production, while in the unit 'E', total pullulan production occurs; thereafter, the fermented broth is taken out from unit 'E' for the downstream processing. It is expected that in the large scale fer-mentation, vessel 'A' and 'D' may automatically increase the temperature up to 38 to 40°C during fermentation due to the higher microbial activity, which is the optima for *A. pullulans* strain presently used. Therefore, at commercial level, heating device will not be required.

The perforation numbers and size have also been evaluated for the segments b^1, b^2 of unit 'B' and 'C'. The results (Tables 3 and 4) clearly indicate that lesser size and numbers reduced the pullulan, and perhaps the biomass transfer from unit 'B' (b^1 and b^2) to the main fermentor might have not be properly released or aeration would have hindered the production process. Furthermore, the larger capacity of the unit 'B' (b^1 and b^2) could favour higher retention of oxygen in the medium and perhaps generated higher pressure by the unit 'B' to push the active cells of *A. pullulans* from the unit 'C' to the main fermentor 'A' which might have facilitated proper aeration to maintain the yeast phase of growth, resulting to higher pullulan production. The low viscosity of the fermentor 'A' could be able to maintain yeast-like cells in the fermentor, and it indicates that the immobilization unit is releasing yeast cells continuously and the rate of biomass transfer from unit 'A' to 'D' could be maintained in turbidostatic conditions, resulting in the constant yield of pullulan (37 ± 1.0 g/l) in several cycles in the scale 1. This result could successfully be achieved in several cycles without disturbing the immobilization unit (Table 6). Further, it is suggested that the size and number of perforations may be increased in b^1, b^2 of unit 'B' and unit 'C', respectively at pilot or industrial scale level. The flow of liquid from the perforations of unit 'B' into the fermentor which could provide proper mixing of the solution in this design as compared to the previous design where b^1 and b^2 lobes were absent in the unit 'B', produced lesser pullulan contents (design and data not shown). Hence, unit 'B' was further modified, having extended lobes b^1 and b^2 with perforations for constant production of pullulan.

The novel fermentor has an aerodynamic design of a multistage continuous fermentor by immobilization of *A. pullulans* cells on polyurethane foam which creates neither back flow of air nor blockage of the fermentor for longer period, because higher viscosity should be restricted in the main fermentor. It has been recorded that the decrease in biomass (yeast-like cells count) decreases the pullulan production (Table 1). Therefore, assessment of yeast-like cells count in the main fermentor can only govern an easy online monitoring for this design. Furthermore, optimization of aeration rate in the unit 'B' is very important because of the necessity of a suitable pressure which may release the cells from immobilization unit 'C' to the main fermentor 'A' to get higher yeast count, resulting in higher pullulan pro-duction, therefore, an optimum quality of air (0.5 vvm) is required for this unit (Table 2). Further, increase in air did not affect the rate of production of the pullulan.

A proper design of a fermentor for immobilization of microbial cells on a suitable solid matrix is the interest of research to find out the maximum production within a short period, involving less energy in order to achieve higher productivity at economical level. Several carrier materials like alginate, carrageenan, γ-alumina, silica gel, etc. are the common support materials used for the immobilization process (Shin et al., 2004; Urkut et al., 2007).

Moreover, there are several limitations of some of the support materials, which do not work properly for a longer

period because sometimes, changes in the pH of the medium during fermentation disturb the cells binding to the support. For example, the phosphate content of the medium disturbs the gelling properties of alginate. Similarly, γ-alumina is disturbed by the change of pH towards the higher side. Hence a non-reactive, stable and non-toxic material should be selected on the basis of the nature of fermentation, metabolite and micro-organisms. Support material used during this study is neither reactive nor toxic, and also not affected by pH, temperature and other chemicals. In this study, the polyurethane foam might have been used as an absorber with better absorption quality having large pores which detached microorganisms by slight pressure exerted by aeration and respired CO_2 and constantly releasing newer/younger active cells from the surface. Thus, it could continuously maintain the turbidostatic condition in the main fermentor, while micro-pores and meso-pores have lesser pore size which did not favour this phenomenon, thereby resulting to lesser production of pullulan and biomass together with the inactive phase of yeast-like cells. However, different quality and quantity of polyurethane foam packing were optimized for immobilization unit 'C'. It has been observed that dense packing reduced the pullulan production. The novel design of the fermentor properly facilitates better aeration, proper mixing of nutrients and reduces the chances of blockage of pores, while single step continuous fermentation affects the immobilization of the cells, resulting in lesser production of the pullulan (data not shown). The interconnected vessels, 'D' and 'E' of higher capacity may be operated on the higher or lower retention period of the fermented broth depending on the tropho- and ido-phase of the fermentation system.

This design is very suitable for pullulan production at economical level, because the immobilization unit 'C' has long lasting ability to produce viable yeast cell phase of growth by this strain of *A. pullulans* without disturbing any other parameter of fermentation in the 18 cycles (Table 6).

Conclusion

A. pullulans cells could efficiently be attached on polyurethane foam of pore size 1000 Á. Our indigenous fermentor design was successfully optimized to achieve continuous and economical production of pullulan up to 18 cycles. The biomass could be continuously generated in the main fermentor with proper concentration of pullulan which improved performance of the fermentation system. Such fermentor could be useful in commercial production of pullulan.

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