3 *Egypt. J. Microbiol.* **43**, pp .37-52 (2008)

Comparative Assessment of Pullulan Production by *Aureobasidium pullulans* under Fed-Batch and Continuous Fermentation

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PULLULAN production was studied in bioreactor under fed-batch and continuous cultures conditions by *Aureobasidium pullulans* ATCC 42023. In fed batch culture, sugar feeding according to consumption rate was the best strategy for pullulan production where it recorded higher pullulan yield than pulsed and continuous sugar feeding. The maximum amount of pullulan concentration was obtained by using sugar feeding according to sugar consumption rate being 88.8 gl^{-1}, after 144 h followed by continuous culture technique at 0.03 h^{-1} dilution rate, being 86.16 gl^{-1} after 96 h of incubation at 28°C. Continuous culture at 0.03 h^{-1} dilution rate, 5.5 pH controlled, aeration rate of 1.0 vvm air and agitation rate of 700 rpm, attained the highest productivity of pullulan being 0.896 $gl^{-1}h^{-1}$. Therefore, continuous culture technique with the dilution rate 0.03 h^{-1} was the most efficient for pullulan production as it increased pullulan formation by 1.5 fold compared with fed-batch culture. There was no remarkable difference between using different solvents for pullulan precipitation on the final concentrations. Produced pullulan gave close physical properties to standard pullulan.

Keywords: *A. pullulans* ATCC 42023, Pullulan production, Bioreactor, Fed-batch culture, Continuous culture

Pullulan is a microbial exopolysaccharide commercially produced by the yeast-like fungus *Aureobasidium pullulans*. The pullulan polysaccharide is a linear homopolysaccharide composed of polymerized glucopyranose units [linked by α (1→4) glucosidic bonds] into maltotriose units [which are joined by α (1→6) glucosidic bonds] (Szymanska & Galas, 1993). Due to its excellent properties, pullulan is used as a low-calorie food ingredient, gelling agent, coating and packaging material for food and drugs, binder for fertilizers and as an oxidation-prevention agent for tablets. Other applications include contact lenses manufacturing, biodegradable foil, plywood, water-solubility enhancer and for enhanced oil recovery (Schuster *et al*., 1993; Israilides *et al*., 1998 and Leathers, 2003). Pullulan production has been the subject of numerous studies conducted under batch (Szymanska & Galas, 1993; Madi *et al*., 1997; Roukas, 1999;

Szymanska *et al*., 1999; Barnett *et al*., 1999 and Lazaridou *et al*., 2002), fed-batch (Ronen *et al.*, 2002) and continuous fermentations (McNeil *et al*., 1989; McNeil & Kristiansen, 1990; Reeslev *et al*., 1993; Simon *et al*., 1993; Audet *et al*., 1996; Gibbs, 1996; Gibbs & Seviour, 1996 and Audet *et al.*, 1998). Lebrun *et al*. (1994) achieved a maximum pullulan concentration of 35 gl^{-1} after 120 h of batch fermentation using a synthetic medium and a 5 l mechanically stirred tank reactor. Gibbs (1996) found that in all impeller systems examined, the increase in agitation rate resulted in a marked and reproducible decrease in exopolysaccharide production by *A. pullulans* under batch conditions in a 10 l stirred tank reactor. McNeil & Kristiansen (1990) studied the influence of temperature on pullulan formation, cell growth and morphology of *A. pullulans* in continuous cultures at 20-36°C on 20 kg/m^3 sucrose using bioreactor. Reeslev *et al*. (1993) studied the yeast-mycelium dimorphism of *A. pullulans* in continuous culture using a defined medium at a constant dilution rate (0.08 h^{-1}). They reported that morphological status of the culture could be controlled by the input concentration of Zn^{2+}. Schuster *et al*. (1993) carried out both batch-wise and continuous fermentations in a stirred vessel fermentor. In batch fermentation, about 45% of glucose was converted into pullulan at maximum formation rate of 0.16 $gl^{-1}h^{-1}$ using standard medium and the yield of pullulan was maintained at 45% in continuous cultures. At a dilution rate of 0.05 h^{-1}, the formation rate of pullulan increased up to 0.35 $gl^{-1}h^{-1}$. Ronen *et al*. (2002) designed a feeding strategy dependent on the culture cellular composition to keep the yeast-like cell concentration high. Feeding was actuated when the yeast-like cell concentration decreased below a threefold. The proposed control strategy improved pullulan production by increasing both productivity and yield of the cells by 67% and 80%, correspondingly.

Many publications are concerned with the control of pullulan synthesis by controlling pullulan fermentation and culture conditions. Overall, this literature is confusing and seemingly contradictory. In part, this could be attributed multiple factors which interact in regulation of pullulan biosynthesis. In this investigation, a comparison between techniques applied for pullulan fermentation, *i.e.*, fed-batch and continuous cultures using bioreactor was thought to optimize pullulan production and identify some factors affecting fermentation processes.

Materials and Methods

Fungal strain

Aureobasidium pullulans ATCC 42023 was obtained from American Type Culture Collection, subcultured monthly on malt agar slants at 30°C, and maintained at 4°C.

Media

Malt agar medium (Atlas, 1997) was used for propagation and preservation of aureobasidium culture. The modified Reeslev & Jensen medium (20% sucrose), (Eltayeb *et al.*, 2005), was used for pullulan production after replacement of its nitrogen source with 7% corn steep liquor.

Standard inoculum

Standard inoculum was prepared by transferring a loop of the tested culture into 250 ml conical flasks containing 50 ml of modified Reeslev & Jensen medium. The inoculated flask was incubated on a rotary shaker at 210 rpm for 48 hr at 30°C**.** The content of this flask was used as a standard inoculum (1 ml contained 6.0–7.0 x 10^5 viable cells). The inoculum was then prepared by centrifugation at 12000 x g for 15 min, and cells were washed twice with sterile distilled water and harvested to inoculate productive medium as the method described by Shabtai & Mukmenev (1995).

Fermentation process and cultural conditions

Fermentation was carried out using a 3 l dished bottom bioreactor Z 6110 / coob (Cole–Parmer Instruments), which consisted of 3 l vessel equipped with lipseal stirrer assembly, automatic pH controller, automatic dissolved O_2, automatic temperature controller, multi–channel peristaltic pump (for feeding) and all the accessories for continuous cultivation. Temperature of pullulan production was controlled at 28°C (Eltayeb *et al.*, 2005). pH of all productive cultures was maintained at 5.5 through cultivation, by the automatic addition of 2 N NaOH. In all experiments, aeration and agitation rates were adjusted at 1 vvm, 700 rpm, respectively.

Fed-batch culture

Three sucrose feeding strategies were conducted to determine the more suitable for high pullulan production.

Continuous and pulsed sugar feeding: The amount of sugar added in both fermentations was equivalent to 200 gl^{-1}. In continuous feeding, sugar solutions (40% sucrose) were fed continuously during the first two, three and four days of incubation at addition rates of 4.15, 2.75 and 2.08 $gl^{-1}h^{-1}$, respectively. In pulsed feeding, four additions of sugar solution were carried out at 24 h intervals during the first 72 h of cultivation period. The pH was controlled at 5.5 with 2 N NaOH solution during the fermentation. The final working volume in the bioreactor was 2 l at the end of feeding period in all cases.

Sugar feeding according to consumption rate: In this feeding strategy, the sugar solution (175 gl^{-1} sucrose) was fed according to consumption rate of sugar obtained from previous study. Thus, addition rate was changed every 24 h incubation period. Applied feeding rates were 0.93, 2.1, 2.0, 1.53, 0.7 and 0.004 $gl^{-1}h^{-1}$, at 24, 48, 72, 96, 120 and 144 h of incubation period, respectively.

Continuous culture

Continuous culture was carried out using 1800 ml of medium. After sterilization, the medium was inoculated by HCDI (approx. 2.5 gl^{-1} dry cells) to accommodate 2 l final working volume. The culture in the vessel was aerated at 1.0 vvm, agitated at 700 rpm and allowed to grow up as a batch culture for 72 hr. After this period, fresh medium was pumped into the culture at different flow

rates of 20, 40, 60 and 80 ml h^{-1} to give 0.01, 0.02, 0.03 and 0.04 h^{-1} dilution rates, respectively. Cultivation of each dilution rate was kept for at least 5 days intervals. Samples were taken aseptically at each steady state to determine the biomass, pullulan production and consumed sugars.

Pullulan recovery

The effects of different organic solvents for pullulan recovery were evaluated to select the best solvent for high recovery. Methanol, ethanol, isopropanol (95 %) and recycled-ethanol were used in this study. A rotary evaporator adjusted at 70°C was used for the distillation of the recycled-ethanol collected from various precipitations.

Pullulan determination

Pullulan was precipitated in the culture supernatant with 2 volumes of ethanol 99%, at 4°C for 1 hr. The precipitate was centrifuged at 4000 x g for 10 min followed by drying at 80°C overnight and was then weighed (GÖksungur *et al*., 2004).

Chemical determinations

Total residual sugars were determined in the fermented liquor according to the method described by Flood & Priestly (1973). Total nitrogen of pullulan was determined using Kjeldahel method as described by Jackson (1973). Organic carbon of pullulan was determined according to the method shown by Jackson (1973). Ash content was determined using the method of Herbert *et al*. (1971).

Parameters related to pullulan production

Yield factor (%), pullulan yield coefficient relative to biomass ($Y_{p/x}$), conversion coefficient (%)**,** pullulan yield (%) and productivity (P) were calculated according to Gamal *et al.* (1991).

Results and Discussion

Comparative assessment of pullulan production efficiency under fed-batch and continuous culture conditions

Fed-batch culture

In fermentation processes where cell growth and/or product formation may be inhibited by high substrate concentration, the substrate was intermittently fed to the culture system in order to maintain a substrate concentration that enhances the biological and metabolic activity. Empirical feeding policies were adopted to get high pullulan productive culture including pulsed and continuous feeding as well as feeding corresponding to sugar consumption rate.

Continuous sugar feeding: Data presented in Tables 1, 2 & 3 show the biological activities of *A. pullulans* ATCC 42023 grown in modified Reeslev & Jensen medium as a fed-batch culture using continuous sucrose feeding rates 4.15, 2.75 and 2.08 $gl^{-1}h^{-1}$ during the first 2, 3 and 4 days of fermentation. Data clearly show that cell dry weight, pullulan concentration and consumed sugar

increased gradually during fermentation period at all feeding rates, although a little variation in final biomass was noticed. The maximum pullulan concentration was obtained after 5, 5 and 6 days of incubation, being 77.1, 82.2 and 85.8 gl^{-1}, at addition rates of 4.15, 2.75 and 2.08 $gl^{-1}h^{-1}$, respectively. The corresponding figures of conversion coefficient were 47, 48.3 and 49.1 %, with pullulan yield of 38.6, 41.1 & 42.9 % and productivity of 0.64, 0.69 and 0.6 gl^{-1} h^{-1}, respectively.

Generally, it appeared that continuous feeding of sucrose at a rate of 2.08 $gl^{-1}h^{-1}$ (during 4 days), was favorable than other feeding rates as shown in Fig. 1.

TABLE 1. Pullulan production by *A. pullulans* ATCC 42023 in modified Reeslev & Jensen medium during 6 days incubation at 28^0C using bioreactor as a fed-batch culture with continuous addition of sucrose at a rate of 4.15 $gl^{-1}h^{-1}$.

Time (hr)	Added solution (ml^{-1})	Added sugar (gl^{-1})	Cell dry weight (gl^{-1})	Pullulan concentration (gl^{-1})	$Y_{p/x}$ (gg^{-1})	Consumed sugar (gl^{-1})	Residual sugars (gl^{-1})	Sugar utilization efficiency (%)	Conversion coefficient (%)	Pullulan yield (%)	Productivity ($gl^{-1}h^{-1}$)
0	0.0	0.0	2.6	-	-	0.0	0.0	-	-	-	-
24	500	100	3.3	7.5	2.3	17.4	82.6	17.4	43.1	7.5	0.31
48	1000	200	5.9	26	4.4	57.8	142.2	28.9	45	13	0.54
72	0.0	0.0	9.3	40.2	4.32	84.8	115.2	42.4	47.4	20.1	0.56
96	0.0	0.0	13.4	57.3	4.3	124.6	75.4	62.3	46	28.7	0.6
120	0.0	0.0	18.1	77.1	4.0	163.8	36.2	81.9	47	38.6	0.64
144	0.0	0.0	19.2	77.3	4	167.3	32.7	83.7	46.2	38.7	0.54

TABLE 2. Pullulan production by *A. pullulans* ATCC 42023 in modified Reeslev & Jensen medium during 6 days incubation at 28^0C using bioreactor as a fed-batch culture with continuous addition of sucrose at a rate of 2.75 $gl^{-1}h^{-1}$.

Time (hr)	Added solution (ml^{-1})	Added sugar (gl^{-1})	Cell dry weight (gl^{-1})	Pullulan concentration (gl^{-1})	$Y_{p/x}$ (gg^{-1})	Consumed sugar (gl^{-1})	Residual sugars (gl^{-1})	Sugar utilization efficiency (%)	Conversion coefficient (%)	Pullulan yield (%)	Productivity ($gl^{-1}h^{-1}$)
0	0.0	0.0	2.8	0.0	-	0.0	0.0	-	-	-	-
24	333.3	66.7	3.6	9.1	2.5	18.8	47.85	28.2	48.4	13.6	0.38
48	666.6	133.3	6.2	26.7	4.3	55.3	78.05	41.5	48.3	20	0.56
72	1000	200	10.3	42.1	4.0	89.2	110.8	44.6	47.2	21.1	0.58
96	0.0	0.0	15.2	60.8	4.0	127.6	72.4	63.8	47.6	30.4	0.63
120	0.0	0.0	18.7	82.2	4.4	170.2	29.8	85.1	48.3	41.1	0.69
144	0.0	0.0	20.1	82.3	4.0	170.9	29.1	85.5	48.2	41.2	0.57

TABLE 3. Pullulan production by *A. pullulans* ATCC 42023 in modified Reeslev & Jensen medium during 6 days incubation at 28^{0}C using bioreactor as a fed-batch culture with continuous addition of sucrose at a rate of 2.08 gl^{-1}h^{-1}.

Time (h)	Added solution (ml^{-1})	Added sugar (gl^{-1})	Cell dry weight (gl^{-1})	Pullulan concentration (gl^{-1})	$Y_{p/x}$ (gg^{-1})	Consumed sugar (gl^{-1})	Residual sugars (gl^{-1})	Sugar utilization efficiency (%)	Conversion coefficient (%)	Pullulan yield (%)	Productivity (gl^{-1}h^{-1})
0	0.0	0.0	2.75	0.0	-	-	-	-	-	-	-
24	250	50	3.1	10.2	3.3	22.4	27.6	44.8	45.5	20.4	0.425
48	500	100	5.5	33.3	6	73.2	26.8	73.2	45.5	33.3	0.69
72	750	150	8.4	56	6.7	121	29	80.7	46.3	37.3	0.78
96	1000	200	12.7	75.3	5.9	157.9	42.1	78.95	47.7	37.7	0.78
120	0.0	0.0	19.3	85.2	4.4	174.6	25.4	87.3	48.8	42.6	0.71
144	0.0	0.0	19.5	85.8	4.4	174.7	25.3	87.35	49.1	42.9	0.6

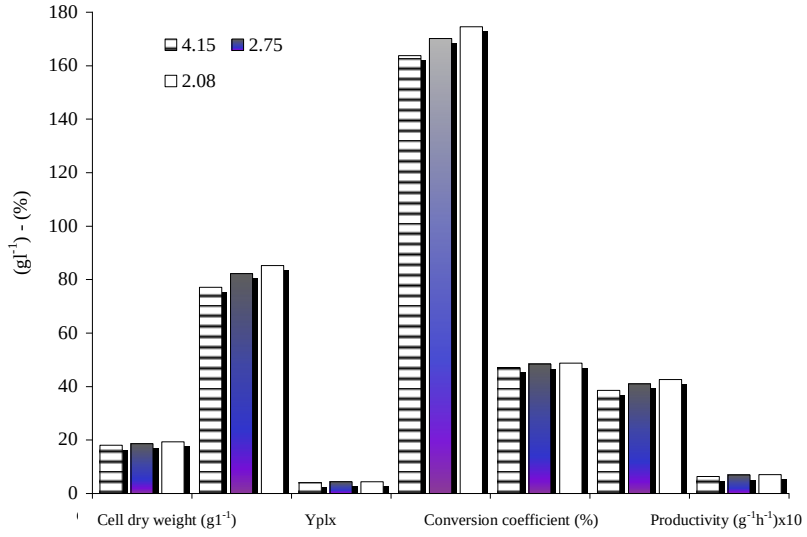

Fig. 1. Pullulan production related parameters as affected by different continuous sugar feeding rates in modified Reeslev & Jensen medium after 5 days of incubation at 28°C using bioreactor as a fed-batch culture.

Pulsed sugar feeding: Pulsed addition of sucrose was carried out every 24 h of incubation during the first 4 days of fermentation, to avoid some problems occurred by continuous addition due to tubes damage, squeezing and blocking with fungal attacks. Data in Table 4 show the effect of pulsed sucrose solution addition at specific addition rate of 0.019 gh^{-1} on growth and pullulan production by *A. pullulans* ATCC 42023. At the end of fermentation period (6 days), the highest figures of cell dry weight, pullulan concentration, sugar utilization efficiency and pullulan yield were obtained being 18.5 gl^{-1}, 77.8 gl^{-1}, 81 % and 38.9 %, respectively. Whereas, the highest productivity was recorded after 5 days being 0.65 $gl^{-1}h^{-1}$.

TABLE 4. Pullulan production by *A. pullulans* ATCC 42023 in modified Reeslev & Jensen medium during 6 days incubation at 28^0C using bioreactor as a fed-batch culture with pulsed addition of sucrose at specific addition rate of 0.019 gh^{-1}.

Time (hr)	Added solution ($ml l^{-1}$)	Added sugar (gl^{-1})	Cell dry weight (gl^{-1})	Pullulan conc. (gl^{-1})	$Y_{p/x}$ (gg^{-1})	Consumed sugar (gl^{-1})	Residual sugars (gl^{-1})	Sugar utilization efficiency (%)	Conversion coefficient (%)	Pullulan yield (%)	Productivity ($gl^{-1}h^{-1}$)
0	250	50	3.1	0.0	-	-	50	-	-	-	-
24	500	100	5.1	7.2	1.41	16.8	33.2	33.6	42.9	14.4	0.3
48	750	150	7.2	24.4	3.39	54.6	45.4	54.6	44.7	24.4	0.5
72	1000	200	9.3	38.3	4.11	87.4	62.6	58.3	43.8	25.5	0.53
96	0.0	0.0	12.7	55.8	4.39	119.3	80.8	59.7	46.7	27.9	0.58
120	0.0	0.0	18.3	77.6	4.24	161.5	38.5	80.75	48	38.8	0.65
144	0.0	0.0	18.5	77.8	4.21	162	38	81	48	38.9	0.54

Sugar feeding according to consumption rate: Sucrose was fed according to consumption rate after reducing initial sugar concentration to match the consumed amount. Thus, addition rate was changed every 24 h of incubation. Data presented in Table 5, indicated that the highest cell dry weight, pullulan concentration and consumed sugar could be obtained after 144 h of incubation, being 19.6, 88.8 and 174.8 gl^{-1}, respectively. Also, the highest figures of pullulan parameters were recorded at the end of fermentation period.

From the above mentioned results, it could be concluded that no great variation on cell dry weight of *A. pullulans* ATCC 42023 was recorded in different feeding strategies of fed-batch culture. However, pullulan concentration and parameters differed from one strategy to another giving the highest and the lowest figures in feeding according to consumed sugar and pulsed addition, respectively, as illustrated by Fig. 2. Generally, it could be stated that continuous feeding according to consumed sugar was the best strategy for using the bioreactor as a fed-batch culture, where pullulan concentration was increased by 1.03 and 1.17 fold than continuous addition of sucrose at a rate of 2.08 $gl^{-1}h^{-1}$ and pulsed sugar feeding, respectively (Tables 3 and 4).

TABLE 5. Pullulan production by *A. pullulans* ATCC 42023 in modified Reeslev & Jensen medium during 6 days incubation at 28^{0}C using bioreactor as a fed-batch culture with different sucrose addition rates according to consumed sugar.

Time (h^{-1})	Added solution ($ml l^{-1}$)	Total added sugar ($g l^{-1}$)	Added sugar ($g l^{-1}$)	Sugar addition rate ($g l^{-1} h^{-1}$)	Celldry weight ($g l^{-1}$)	Pullulan conc. ($g l^{-1}$)	$Y_{p/x}$ ($g g^{-1}$)	Consumed sugar ($g l^{-1}$)	Residual sugars ($g l^{-1}$)	Sugar utilization efficiency (%)	Conversion coefficient (%)	Productivity ($g l^{-1} h^{-1}$)
0	0.0	0.0	0.0	0.0	2.77	0.0	0.0	0.0	0.0	0.0	0.0	0.0
24	128	22.5	22.5	0.93	3.3	13.3	4	22.5	0.0	100	59.1	0.55
48	290.2	73.3	50.8	2.1	5.3	40.8	7.7	73.3	0.0	100	55.7	0.85
72	273.1	121	47.9	2	8.6	57.5	6.7	120.6	0.4	99.6	47.7	0.8
96	210.9	157.9	36.9	1.53	13.1	77.3	5.9	157.4	0.5	99.7	49.1	0.81
120	96.4	174.6	16.8	0.7	19.4	84.2	4.3	174.3	0.3	99.8	48.3	0.7
144	1.0	175	0.1	0.004	19.6	88.8	4.5	174.8	0.2	99.9	50.8	0.62

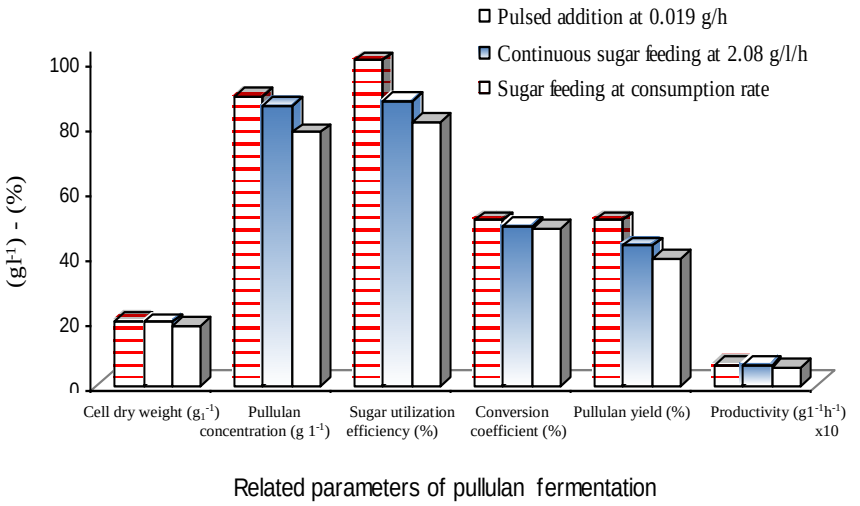

Fig. 2. Pullulan production parameters by *A. pullulans* ATCC 42023 as affected by different sugar feeding strategies in modified Reeslev & Jensen medium after 6 days of incubation at 28°C using bioreactor as a fed-batch culture.

Continuous culture

The biological activity of *A. pullulans* ATCC 42023 on modified Reeslev & Jensen medium was studied in continuous culture at different dilution rates (different steady states). Pullulan production, consumed sugar, biomass dry

weight, and productivities were expressed as a function of dilution rates. Production medium was added to the bioreactor at different flow rates ranged from 20 to 80 ml h^{-1}, after 72 h of incubation.

Results in Tables 6, 7, 8 & 9 and illustrated by Fig. 3 show that varying the dilution rates, resulted in changing the steady state situation of pullulan yield, conversion coefficient and pullulan productivity. At steady state levels of 0.01, 0.02, 0.03 & 0.04 h^{-1}, pullulan production, consumed sugar and cell density remained constant for five days, while washing out was observed at 0.04 h^{-1}. Mean values of the highest amount of pullulan concentration (86.16 gl^{-1} or 5.17 gh^{-1}) was attained at 0.03 h^{-1}. Pullulan concentration and cell dry weight out let ranged from 83 – 84.1 gl^{-1} and 18.8 – 19.4 gl^{-1}, respectively for steady state 0.02 h^{-1}, whereas they ranged from 80 – 81.3 gl^{-1} and 19.8 – 20.4 gl^{-1} at 0.01 h^{-1}. At dilution rate 0.04 h^{-1}, where no steady state was observed, pullulan out let, and biomass in bioreactor were decreased from 60.1 to 8.2 and from 18 to 4.1 gl^{-1}, respectively during five days of incubation. With respect to consumed sugar (gh^{-1}) at different dilution rates, it was found that sugar consumption rate increased with the increase in sugar input up to 0.03 h^{-1}. The amount of sugar input was 3.5, 7.0, 10.5, and 14 gh^{-1} for 0.01, 0.02, 0.03 and 0.04 h^{-1} dilution rate, respectively. Pullulan yield, conversion coefficient and pullulan productivity, were increased with increasing dilution rate (sugar input), reaching their maximum level at 0.03 h^{-1} being 49.22, 51.3% and 0.896 $gl^{-1}h^{-1}$, respectively then decreased at 0.04 h^{-1} dilution rate. Therefore, it could be stated that the maximum dilution rate to be used, is 0.03 h^{-1} to give maximum pullulan production by *A. pullulans* ATCC 42023.

TABLE 6. Pullulan production by *A. pullulans* ATCC 42023 in modified Reeslev & Jensen medium as continuous culture at 0.01 h^{-1} dilution rate (20 ml medium / hr flow rate / 2000 ml culture).

Time in days	Sugar in put (gh^{-1})	Sugar out let (gh^{-1})	Consumed sugar (gl^{-1})	Consumed sugar (gh^{-1})	Cell dry weight out let (gl^{-1})	Pullulan conc. out let (gl^{-1})	Pullulan conc. out let (gh^{-1})	Pullulan yield (%)	Conversion coefficient (%)	Productivity ($gl^{-1}h^{-1}$)
1	3.5	0.03	173.5	3.47	19.8	80	1.6	45.7	46.1	0.83
2	3.5	0.035	173.25	3.465	20.1	80.5	1.61	46	46.5	0.839
3	3.5	0.04	173	3.46	20.2	79.6	1.59	45.5	46	0.829
4	3.5	0.031	173.5	3.47	20.4	80	1.6	45.7	46.1	0.833
5	3.5	0.034	173.3	3.51	20	81.3	1.62	46.5	46.9	0.847
Means	3.5	0.034	173.31	3.475	20.1	80.28	1.604	45.88	46.32	0.8356

TABLE 7. Pullulan production by *A. pullulans* ATCC 42023 in modified Reeslev & Jensen medium as continuous culture at 0.02 h^{-1} dilution rate (40 ml medium / hr flow rate / 2000 ml culture).

Time in days	Sugar in put (gh^{-1})	Sugar out let (gh^{-1})	Consu-med sugar (gl^{-1})	Consu-med sugar (gh^{-1})	Cell dry weight out let (gl^{-1})	Pullulan conc. out let (gl^{-1})	Pullulan conc. out let (gh^{-1})	Pullulan yield (%)	Conversion coefficient (%)	Productivity ($gl^{-1}h^{-1}$)
1	7.0	0.1	172.5	6.9	18.8	83	3.3	47.4	48.1	0.865
2	7.0	0.13	171.75	6.87	19.3	83.2	3.3	47.5	48.4	0.867
3	7.0	0.14	171.5	6.86	19.4	84	3.3	48	49	0.875
4	7.0	0.1	172.5	6.9	19.2	83.5	3.34	47.7	48.4	0.87
5	7.0	0.16	171	6.84	19.0	84.1	3.4	48	49.2	0.88
Means	7	0.126	171.85	6.874	19.14	83.56	3.328	47.72	48.62	0.8714

TABLE 8. Pullulan production by *A. pullulans* ATCC 42023 in modified Reeslev & Jensen medium as continuous culture at 0.03 h^{-1} dilution rate (60 ml medium / h flow rate / 2000 ml culture).

Time in days	Sugar in put (gh^{-1})	Sugar out let (gh^{-1})	Consumed sugar (gl^{-1})	Consu-med sugar (gh^{-1})	Cell dry weight out let (gl^{-1})	Pullulan conc. out let (gl^{-1})	Pullulan conc. out let (gh^{-1})	Pullulan yield (%)	Conversion coefficient (%)	Productivity ($gl^{-1}h^{-1}$)
1	10.5	0.4	168.3	10.1	18.2	86.1	5.17	49.2	51.2	0.89
2	10.5	0.42	168	10.08	18.0	86.2	5.17	49.3	51.3	0.9
3	10.5	0.43	167.8	10.07	18.3	85.8	5.15	49	51.1	0.89
4	10.5	0.4	168.3	10.1	18.7	86.2	5.17	49.2	51.2	0.9
5	10.5	0.41	168.2	10.09	18.6	86.5	5.19	49.4	51.4	0.9
Means	10.5	0.412	168.12	10.08 8	18.36	86.16	5.17	49.22	51.3	0.896

TABLE 9. Pullulan production by *A. pullulans* ATCC 42023 in modified Reeslev & Jensen medium as continuous culture at 0.04 h^{-1} dilution rate (80 ml medium / h flow rate / 2000 ml culture).

Time in days	Sugar in put (gh^{-1})	Sugar out let (gh^{-1})	Consumed sugar (gl^{-1})	Consumed sugar (gh^{-1})	Cell dry weight out let (gl^{-1})	Pullulan conc. out let (gl^{-1})	Pullulan conc. out let (gh^{-1})	Pullulan yield (%)	Conversion coefficient (%)	Productivity ($gl^{-1}h^{-1}$)
1	14	3.7	128.75	10.3	18.0	60.1	4.8	34.3	46.7	0.63
2	14	6.2	97.5	7.8	14.2	47.4	3.79	27	48.6	0.49
3	14	9.3	58.75	4.7	9.7	27.4	2.19	15.6	46.6	0.29
4	14	10.7	41.25	3.3	7.2	15.1	1.2	8.6	36.6	0.16
5	14	12.4	20	1.6	4.1	8.2	0.7	4.7	41	0.09
Means	14	8.46	69.25	5.54	10.64	31.64	2.516	18.04	43.9	0.332

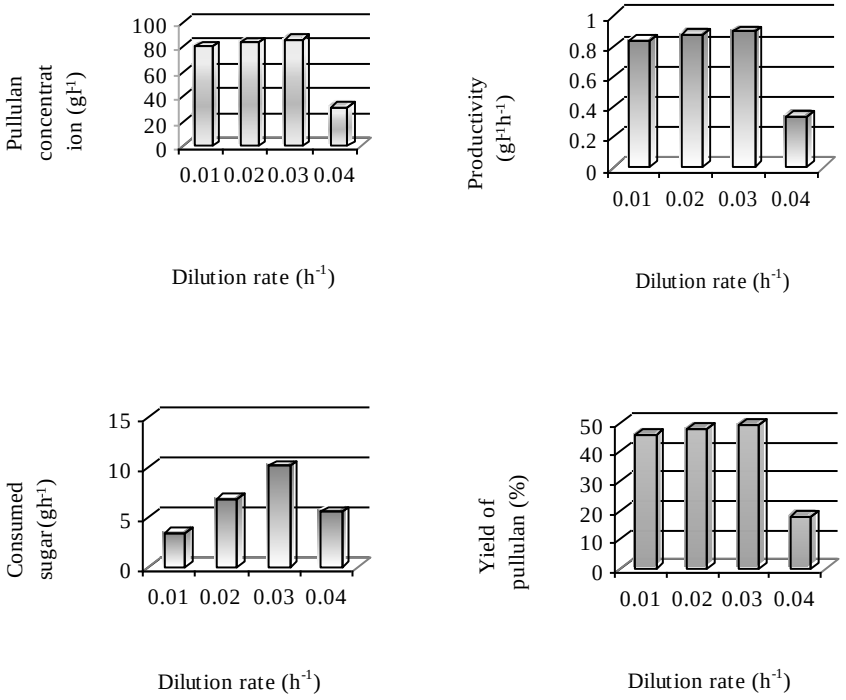

Fig. 3. Pullulan concentration, consumed sugar, productivity and pullulan yield by *A. pullulans* ATCC 42023 in modified Reeslev & Jensen medium at 28°C using bioreactor as continuous culture at different dilution rates.

Comparing the amount of pullulan and pullulan production parameters obtained by *A. pullulans* ATCC 42023 using both fermentation techniques (as in Table 10), it could be noticed that the highest productivity was attained by using continuous culture at 0.03 h^{-1} dilution rate being 0.896 $gl^{-1}h^{-1}$. While the maximum amount of pullulan was obtained from fed-batch bioreactor technique being 88.8 gl^{-1}, after 144 h followed by continuous culture technique at 0.03 h^{-1} dilution rate, being 86.16 gl^{-1} after 96 h. If the production period of 0.03 h^{-1} steady state of continuous culture technique was maintained to equal that of fed-batch bioreactor (144 h), pullulan production under continuous culture technique will increase by 1.5 fold than that of fed-batch bioreactor technique. Therefore, it is noteworthy to state that the continuous culture t0.03 h^{-1} dilution rate is the best method for pullulan production by cultivation of *A. pullulans* ATCC 42023 in modified Reeslev & Jensen medium. In this respect, Schuster *et al.* (1993) found that dilution rate ranged from (0.04 - 0.05 h^{-1}) gave the highest pullulan production, where the formation rate of polysaccharide increased up to 0.35 $gl^{-1}h^{-1}$. Whereas, Reeslev *et al.* (1993) found that at a constant dilution rate of 0.08 h^{-1}, higher pullulan could be produced.

TABLE 10. Comparative data of pullulan fermentation in fed-batch and continuous cultures.

Technique results	Fed-batch bioreactor with sugar feeding at consumption rate	Continuous fermentation at different dilution rates (D) with pH controlled at 5.5			
		0.01 (h^{-1})	0.02 (h^{-1})	0.03 (h^{-1})	0.04 (h^{-1})
Pullulan concentration (gl^{-1})	88.8	80.28	83.56	86.16	31.64
Productivity ($gl^{-1}h^{-1}$)	0.62	0.836	0.87	0.896	0.33
Pullulan yield (%)	50.7	45.88	47.72	49.22	18.04
Conversion coefficient (%)	50.8	46.32	48.62	51.3	43.9
Production time (hr)	144	96	96	96	96

Pullulan recovery

Data in Table 11 indicate that there was no remarkable difference between pullulan concentrations obtained by using methanol or ethanol for precipitation. Nevertheless, it is recommended to use methanol for precipitation when recovering non-food grade pullulan. Additionally, methanol is cheaper than ethanol or isopropanol for long time use in precipitation. Data also indicated that reusing of distilled ethanol for pullulan precipitation had the same efficiency of extraction, but it only decrease pullulan concentration by about 1.1% compared with first-time ethanol application. However, recycling of ethanol may some how reduces the production costs of pullulan production.

TABLE 11. Pullulan recovery using different organic solvents.

Organic solvent	Pullulan concentration (gl^{-1})
Methanol (95 %)	88.5
Ethanol (95 %) (control)	88.2
Distilled ethanol from previous precipitations	87.2
Isopropanol (95 %)	86.4

Physicochemical properties of produced pullulan

The physicochemical properties of standard pullulan and the pullulan produced during continuous process are given in Table 12. Pullulan produced by *A. pullulans* ATCC 42023 gave close physical properties of standard pullulan. However, lower carbon content was recorded in produced pullulan being 38.6 %. Produced pullulan also contained higher amount of total nitrogen and ash being 0.17 % and 0.21 %, respectively.

TABLE 12. A comparison between physicochemical properties of standard pullulan and pullulan produced by *A. pullulans* ATCC 42023 under continuous fermentation.

Physicochemical properties	Standard pullulan specifications	Specifications of produced pullulan
Appearance	White powder	White powder
Solubility	Soluble in water	Soluble in water
Carbon content	42 %	38.6 %
Total nitrogen	0.09 %	0.17 %
Ash	0.1 %	0.21 %

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(*Received* 17/9/2007;
accepted 26/5/2008)

التقييم المقارن لإنتاج البوليولان بواسطة فطر *Aureobasidium pullulans* تحت نظامي التنمية بالدفعة الواحدة المغذاه و المزرعة المستمرة

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أجرى هذا البحث لدراسة إنتاج البوليولان بواسطة السلالة *Aureobasidium pullulans* ATCC 42023 بتنميتها في المخمر بنظام الدفعة الواحدة المغذاه ونظام المزرعة المستمرة. حيث وجد أن إضافة السكر تبعاً لمعدل استهلاكه كانت أفضل طريقة للتنمية بنظام الدفعة الواحدة المغذاه , نظراً لزيادة محصول البوليولان مقارنة بالتنمية بنظام الدفعة الواحدة المغذاه المستمرة أو المتقطعة. و تم الحصول على أعلى تركيز للبوليولان الناتج عند التنمية بنظام الدفعة الواحدة المغذاه تبعاً لمعدل استهلاك السكر (٨٨,٨ جم/لتر) بعد ١٤٤ ساعة من التحضين, متبوعاً بالتنمية بنظام المزرعة المستمرة عند معدل تخفيف ٠,٠٣ / ساعة (٨٦,١٦ جم/لتر) بعد ٩٦ ساعة من التحضين على درجة حرارة ٢٨°م. بينما تم الوصول إلى أعلى إنتاجية للبوليولان (٠,٨٩٦جم/لتر/ساعة) عند معدل تخفيف ٠,٠٣/ ساعة في نظام التنمية بالمزرعة المستمرة وعند حموضة ثابتة مقدارها ٥,٥ ،معدل تهوية مقداره ١,٠ vvm ومعدل تقليب مقداره ٧٠٠ لفة/ق. و تعتبر التنمية بنظام المزرعة المستمرة بمعدل تخفيف ٠,٠٣ /ساعة هي الأنسب من بين طرق الإنتاج المختبرة الأخرى حيث أنها أدت إلى زيادة انتاج البوليولان ١,٥ ضعفاً مقارنة بنظام التنمية ذات الدفعة الواحدة المغذاه .كما لوحظ أنه لا يوجد اختلاف كبير في كمية البوليولان الناتج عند الترسيب بالمذيبات العضوية المختلفة. و يتميز البوليولان الناتج بنظام المزرعة المستمرة بصفاته الفيزيائية القريبة من البوليولان القياسي .