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Application of different feeding strategies in fed batch culture for pullulanase production using sago starch

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ABSTRACT

The production of pullulanase by *Bacillus flavothermus* KWF-1 in batch and fed batch culture were compared using 2 L bioreactor. In batch culture, 0.0803 U/mL of pullulanase activity with specific activity of 0.0213 U/mg was produced by controlling the agitation speed and temperature at 200 rpm and 50 °C, respectively. Fed batch production was studied by feeding the culture with different sago starch concentrations in various feeding modes for enhanced pullulanase production. Exponential feeding mode at dilution rate of 0.01/h was the preeminent strategy for enhanced pullulanase production of 0.1710 U/mL with specific activity of 0.066 U/mg. It had shown an increment of pullulanase production and specific activity by 2.1 and 3.1-fold, respectively when compared to batch culture. Increment of pullulanase activity in exponential feeding mode improved hydrolyzation of sago starch into maltotriose and panose by 4.5 and 2.5-fold respectively compared to batch system.

1. Introduction

Ability of pullulanase to hydrolyze the branch points in various starch materials other than pullulan broadens the application of the enzyme in starch processing industries (Norman, 1979). Pullulanase (EC 3.2.1.41) is a debranching enzyme that possesses the specific ability to hydrolyze the α-1,6 linkage in pullulan and branched polysaccharides (Gomes, Gomes, & Steiner, 2003; Hii, Ling, Ariff, & Rosfarizan, 2009a; Kuroiwa, Shoda, Ichikawa, Sato, & Mukataka, 2005; Zhang & Jin, 2011). In industries, pullulanase is used in combination with glucoamylase or β-amylase in the saccharification process to produce glucose or maltose syrup (Kang et al., 2011). Pullulanase production in batch culture has been extensively reported compared to fed batch approach. *Metroxylon sagu* or commonly known as sago or "rumbia" have tremendous application in many industries. Native sago starch contains about 24–30% amylose and 70–76% amylopectin (Mohamed, Jamilah, Abbas, Abdul Rahman, & Roselina, 2008; Nakamura, 1996; Wong et al., 2007). Sago is cheap, easy to gelatinize, highly viscous if properly extracted and easily moulded (Fasihuddin, Peter, Jean, Sylvie, & Alain, 1999). In this research, sago starch was utilized as limiting substrate and feeding material for pullulanase production in fed batch culture. Utilization of starch as a carbon source has been proven to give higher yield of pullulanase compared to other carbon sources (Hii, Ling, Rosfarizan, & Ariff, 2009b).

Increase in health awareness has directly affected the food industries. Demand for unique sugars such as maltotriose and panose have increased. Maltotriose has many excellent properties such as mild sweetness, ability to retain moisture, prevention of retrogradation of starch in foodstuffs, less colour formation compared with maltose syrups, glucose syrups or sucrose, good heat stability and low viscosity. These properties are useful in both food and pharmaceutical industries (Singh, Saini, & Kennedy, 2010a; Singh, Saini, & Kennedy, 2010b; Wu, Chen, Tong, Xu, & Jin, 2009; Zoebelein & Bollert, 2001). Panose is a trisaccharide constituted by a maltose molecule bonded to a glucose molecule by an α-1,6-glycosidic bond. Panose is known as pre-biotic carbohydrate due to its ability to promote the proliferation of microorganisms of the genus *Difidobacterium* and *Lactobacillus* in human intestines and also to inhibit the growth of undesirable microorganisms such as *Salmonella* and *Escherichia coli* (Chung & Day, 2002; Chung & Day, 2004; Fernandes & Rodrigues, 2006; Machida, Fukui, & Komoto, 1986). Panose can be used as anti-fading agents for food pigments, food antioxidant and sweetener besides being anti-carcinogenic (Higashimura, Emura, Kuze, Shirai, & Koda, 2002; Miyake, Mikihiko, & Kano, 1985). Maltose syrup is also widely used in food and

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pharmaceutical industries due to its mild sweetness, good thermal stability, low viscosity in solution, and lack of colour formation (Govindasamy, Campanella, & Oate, 1995; Li, Bai, Mousaa, Zhang, & Shen, 2011; Li, Zou, et al., 2011).

Fed batch is an evolutionary process to replace batch culture as it can extend product formation stage besides helping to reduce medium viscosity and eliminate repressive effects of rapidly utilized carbon sources. An experiment conducted by Kuo, Lin, Chen, Lin, and Duan (2009) to study the effect of fed-batch culture in the production of cyclodextrion glucanotransferase (CG) by alkaliphile *Bacillus* sp showed that CG production was 360% higher in fed batch culture compared to batch culture. The objective of this research was to implement fed batch system into the production of thermostable pullulanase. Fed batch system with different feeding strategies using sago starch as sole carbon source and *Bacillus flavothermus* KWF-1 were applied to optimize the production of thermostable pullulanase, which functions optimally at 70–80 °C.

2. Materials and methods

2.1. Substrate and pretreatment

2.1.1. Sago starch pretreatment

Gelatinized sago starch was used as substrate. Raw sago starch was pretreated using heat to form gelatinized starch. The raw sago starch was gelatinized using water bath system controlled at 100 °C. Gelatinized sago starch was then mixed with other medium components.

2.1.2. Pullulan-peptone yeast extract agar medium (Pullulan-PYE agar)

B. flavothermus KWF-1 from stock culture was grown on Pullulan-peptone yeast extract agar (pullulan-PYE agar) to obtain single colony. The medium consist of 0.5% (w/v) pullulan, 0.03% (w/v) KH_2PO_4, 1.0% (w/v) peptone, 0.3% (w/v) yeast extract, 0.267%(w/v) $Na_2HPO_4{\cdot}12H_2O$, 0.02% (w/v) $MgCl_2{\cdot}6H_2O$, 0.1% (w/v) NH_4Cl and 2.0% (w/v) agar. The initial pH of the medium was adjusted to 7.0. Medium was autoclaved at 121 °C for 15 min.

2.1.3. Peptone yeast extract production medium (PYE)

Peptone yeast extract (PYE) liquid medium was used for the growth and production of pullulanase by *B. flavothermus* KWF-1 culture. The medium consisted of 2.0% (w/v) sago starch, 1.75% (w/v) peptone, 0.5% (w/v) yeast extract, 0.1% (w/v) KH_2PO_4 and 0.02% (w/v) $MgSO_4{\cdot}7H_2O$. The initial pH was adjusted to 7.0. Medium was autoclaved at 121 °C for 15 min.

2.2. Enzyme production

2.2.1. Microorganisms and inoculum preparation

B. flavothermus KWF-1 was previously isolated from a local hot spring environment and grown at 50 °C in peptone yeast extract (PYE) medium (Subash Nair, Rekha Singhal, & Madhusudan Kamat, 2006). The pH of the medium was adjusted to 7.5 using 0.1 M NaOH. *B. flavothermus* KWF-1 was cultured in 250 mL conical flasks and incubated overnight in an orbital shaker at 50 °C and 200 rpm. The cells were then centrifuged at 4000 rpm for 10 min, washed once with 0.85% (w/v) saline solution to give an optical density (OD) of 0.6–0.8 at 550 nm.

2.2.2. Detection of pullulanase producer by using pullulan-PYE agar

The pullulan-PYE agar formulation was prepared as mentioned in Section 2.1.2. The pullulanase producer was determined by pullulan precipitation technique using methanol (Bibel et al., 1998). The plate was flooded twice with methanol and left for 1 h. A clear halo zone around the bacteria colony appeared against a turbid background if the bacteria produced pullulanase. To view pullulanase degradation below the colonies, the colonies on the plate was scraped and the agar was flooded again with methanol. In order to obtain better resolution of the degraded area or clearing zone, pullulan-PYE plate was flooded with 0.1% (w/v) of congo red solution for 15 min and then washed several times with 1 M of NaCl to increase the bonding of the dye to pullulan (Ruijssenaars & Hartmans, 2001). Plate was then washed with distilled water several times to remove unbound dyes.

2.2.3. Production of pullulanase in batch culture

Batch culture was initiated in 700 mL of PYE medium consisting of 2% (w/v) sago starch. The medium was inoculated with 10% (v/v) of starter culture. The incubation temperature of the bioreactor was controlled at 50 °C with agitation speed of 200 rpm and supplied with constant aeration for the production of pullulanase.

2.2.4. Bioreactor set-up

Fermentations were conducted in 2 L bioreactor (with working volume of 1.5 L, Biostat® B, Germany) supplied by Sartorius BBI System GmbH. The culture vessel was double jacketed borosilicate glass. The internal concave bottom ensures optimum culture mixing even at low stirring speeds. The bioreactor was equipped with control module unit, peristaltic pump, motor, temperature sensor, air sparger, impeller, pH-electrode, pO_2-electrode, antifoam probe and level probe. A sterilizable polagraphic pO_2-electrode (Mettler Toledo AG, Switzerland) and glass pH electrode (Mettler Toledo) were used to measure the dissolved oxygen (DO) level and pH of the culture, respectively. The bioreactor was fixed with two six-bladed disc impellers on the stirrer shaft to provide sufficient agitation throughout the fermentations.

The production medium was prepared in the vessel (according to Section 2.1.3). The PYE medium was autoclaved at 121 °C for 15 min. Sufficient oxygen was maintained in the production medium by continuous supply of oxygen. Bacterial inoculum of 10% (v/v) was inoculated into the production medium. In all fermentations, the temperature of the vessel was controlled at 50 °C and agitation speed was fixed at 200 rpm.

2.2.5. Fed batch operation

Fed-batch fermentation was carried out using the same fermentor and conditions as mentioned in Section 2.2.4. The fermentation was instigated as batch mode in 700 mL of PYE medium. The fed batch system was initiated when the culture reached quasi-steady state where the growth limiting substrate depleted ($S = 0$) and maximum biomass production was achieved ($X = X_{max}$) in the fermentation medium. Fed batch system was performed by supplying limiting substrate into the vessel using external controlled peristaltic pump (Econo Gradient Pump from Bio-Rad).

Fix mode, variable constant and variable exponential feeding strategies was used for pullulanase production in fed batch culture. For fix mode feeding method, high concentration of growth limiting substrate was added to culture in undiluted form. The feeding medium was provided at a constant rate during intervals when the limiting substrate in the fermentation vessel was exhausted ($S = 0$).

In variable constant feeding mode, the feed rate (F) of growth limiting substrate was provided at constant rate continuously into the culture until the maximum level of vessel was reached according to Eq. (1). Dilution rate was set below μ_{max} value in order to permit high consumption of substrate once it enters the vessel. In this type of fed batch culture, the dilution rate changes with time. Although the total biomass (XV) increases with time (t), but the cell concentration (X), remains constant ($dx/dt = 0$) and therefore $\mu = D$. This situation is termed a quasi-steady state. As

the feeding progressed, the dilution rate decreases and vessel volume increases (Peter et al., 1995).

$$D = \frac{F_i}{V_{(0)} + F_{it}} = \frac{F_i}{V_{(t)}} \quad (1)$$

where, D = dilution rate; F_i = feed rate (mL/min); $V_{(0)}$ = initial volume of the culture in the vessel (L); $V_{(t)}$ = volume of the culture at time (L).

Application of exponential fed batch culture was able to prolong the rapid growth of microorganism in exponential phase by supplying the culture with substrate at a suitable rate. In order to maintain the growth limiting substrate level at low level, the value of μ must be kept lower than μ_{max}. This condition will prevent accumulation of excessive growth limiting substrate in the culture due to rapid utilization of substrate during fermentation process. Feeding rate (F_i) was increased exponentially with time and F_i at time t is expressed as illustrated in Eq. (2).

$$F_{i(t)} = F_{i(0)} e^{\mu t} \quad (2)$$

where, $F_{i(t)}$ = feed rate at time (mL/min); $F_{i(0)}$ = feed rate at the beginning (mL/min); μ = dilution rate (D).

2.3. Analytical methods

2.3.1. Sample preparation

The fermentation medium was withdrawn at 4 h intervals and centrifuged at 4000 rpm for 20 min. Pellet was set aside for biomass determination while the supernatant was used for further analysis.

2.3.2. Detection of maltose, maltotriose and panose

High performance liquid chromatography was used to quantify the amount and type of sugars in supernatant. Maltose, maltotriose and panose standards were purchased from Sigma Aldrich. HPLC analysis was performed using Agilent 1100 series HPLC with Zorbax Carbohydrate Analysis Column (5.0 μm, 4.6 mm × 250 mm). Mobile phase used were Acetonitrile and water at ratio of 70:30. The flow rate and the column temperature were maintained at 1.0 mL/min and 40 °C respectively.

2.3.3. Pullulanase assay

Pullulanase activity was assayed via dinitrosalicylic (DNS) method. The reaction mixture containing 1 mL supernatant (crude enzyme) and 1 mL of 1% (w/v) pullulan in 100 mM glysine–NaOH buffer pH10.0 was incubated at 80 °C for 30 min. DNS reagent (1 mL) was added into the mixture (1 mL) followed by 60 μL 0.1 M NaOH. The mixture was then incubated at 100 °C for 5 min and then cooled in ice. Distilled water (10 mL) was added to the mixture and the colour intensity was measured at 540 nm. One unit of pullulanase activity was defined as the amount of pullulanase required to catalyze the formation of 1 μmol/min reducing sugar under this assay condition (Miller, 1959).

2.3.4. Protein determination

Determination of protein content in sample was conducted based on modified Lowry's method using bovine serum albumin (BSA) as the standard protein (Waterborg & Matthews, 1994).

2.3.5. Biomass determination

Dry cell weight measurement was performed for determination of cell biomass. Cellulose acetate of 0.2 μm membrane pore size was dried in an oven at 70 °C to constant weight. Pellet that was collected from the sample was dissolved with 1 mL of distilled water and 30 μL of α-amylase. Addition of α-amylase was important to degrade starch in the sample. The mixture was incubated at 100 °C for 10 min. After incubation, the mixture was poured into the holding reservoir fitted on the pre-weighed dry filter membrane. The filter membranes with biomass were dried in the oven at 70 °C and weighed several times until there was no fluctuation in the weight.

3. Results and discussion

3.1. Qualitative detection of pullulanase production in pullulan-PYE agar

Ability of *B. flavothermus* KWF-1 to degrade pullulan by production of pullulanase was detected by growing the culture on pullulan-PYE agar. In pullulan precipitation method, ethanol was used as a precipitation agent. The precipitated pullulan on agar medium formed a turbid white precipitation. The clearing zone formed around the colonies indicates that the degraded pullulan by the action of pullulanase is produced by *B. flavothermus* KWF-1. The colonies were then scraped off the agar and flooded again with methanol to obtain view on pullulanase degradation area below the colonies (Fig. 1a).

To obtain better contrast of the clearing zone, pullulan-PYE agar plate with colonies were flooded with 0.1% (w/v) congo red (CR) solution. CR dye is used to screen the presence of several types of enzymes such as xylanase and cellulose due to its ability to form non-covalent bonding with polysaccharides (Ruijssenaars & Hartmans, 2001). Maubasher, Wahsh, and El-Kassem (2010) recently reported the application of CR for pullulanase detection. This dye-polysaccharide interaction helps to make the non-degraded pullulan more visible on the plate. Initially, the plate was flooded with 0.1% (w/v) of CR solution for 15 min. The plate was then washed several times with 1 M of NaCl to increase the bonding of the dye to pullulan. Subsequently, it was washed with distilled water several times to remove unbound dyes. Fig. 1c shows clearing zones formed around the colonies. At the area where pullulan was degraded, dye was unable to attach resulting in clearing zones. As shown in Fig. 1b and d, it is an addition of 1 M HCl that changed the red background into blue (Ruijssenaars & Hartmans, 2001). This will further increase the contrast as the background of the plate will turn dark blue, resulting in clearer observation of pullulanase activity. Fig. 1b clearly illustrates the complete utilization of pullulan below the colonies. The colonies were scrapped off to obtain a better picture of degraded pullulan below the colonies on the agar plate. The image shows that *B. flavothermus* KWF-1 utilized the nearest pullulan available for growth.

Application of CR had been widely used for detection of several cellulolytic enzymes such as cellulase and xylanase. Pullulanase detection will usually be performed using ethanol precipitation. However, application of CR for pullulanase detection resulted in better resolution of degradation area on pullulan agar by the colour contrast. Thus, in future this method can be used to detect pullulanase activity on pullulan agar instead of using ethanol precipitation method which results in poor contrast between the clearing zone and the background. Increase of the clearing zone visibility will also aid the measurement of it.

3.2. Production of pullulanase in batch culture

Preliminary study of pullulanase production by *B. flavothermus* KWF-1 batch culture was performed in 2 L bioreactor. Fig. 2a and b represents the kinetics of pullulanase production in batch culture. The specific growth rate (μ) of *B. flavothermus* KWF-1 was 0.0327/h with maximum specific growth (μ_{max}) of 0.0654/h. The maximum biomass concentration of 2.67 g/L was achieved after 76 h of incubation when cell enters the stationary phase. In a research conducted by Hii et al. (2009b) for pullulanase production using various starch materials, strain *Raoultella planticola* DSMZ 4617

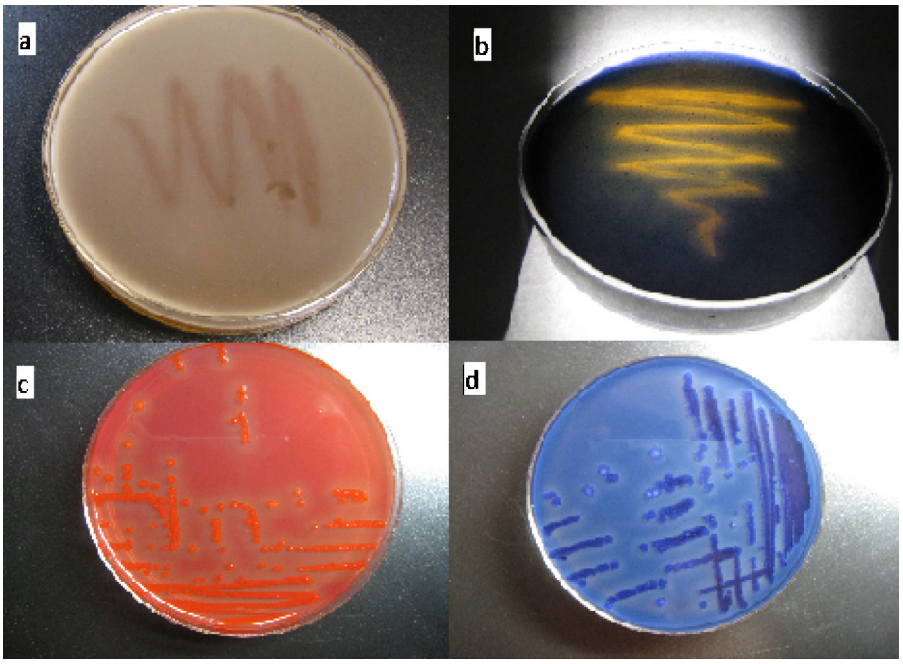

Fig. 1. Degradation of pullulan by *Bacillus flavothermus* on PYE medium. (a) Ethanol precipitation method. (b) Colonies removed. (c) Using congo red. (d) Congo red + HCl.

was reported to enter stationary phase after 48 h. The delayed time to reach stationary phase in current experiment can be explained by the extended time required by *B. flavothermus* KWF-1 to surpass the adaptation phase. Based on Fig. 2a, increase of pullulanase activity collectively with biomass concentration at exponential stage indicates the pullulanase production was growth-associated. However, starting from 84 h of incubation, pullulanase activity decreases although there was a slight increase in biomass concentration. Thus, pullulanase production by *B. flavothermus* KWF-1 is both growth and non-growth associated.

Highest pullulanase activity of 0.0803 U/mL with maximum specific activity of 0.0213 U/mg was detected at early stationary phase (t = 76 h). Similar observation was reported by Subash Nair et al. (2006) whereby maximum pullulanase production by *Bacillus cereus* FDTA-13 was also obtained at early stationary phase. However, in a different research by Mrudul, Reddy, and Seenayya (2011), maximum pullulanase production which was growth and non-growth associated by *Clostridium thermosulfurogenes* SVM 17 was reported to occur at latter part of stationary phase. Absence of pullulanase activity can be observed at early stage of fermentation.

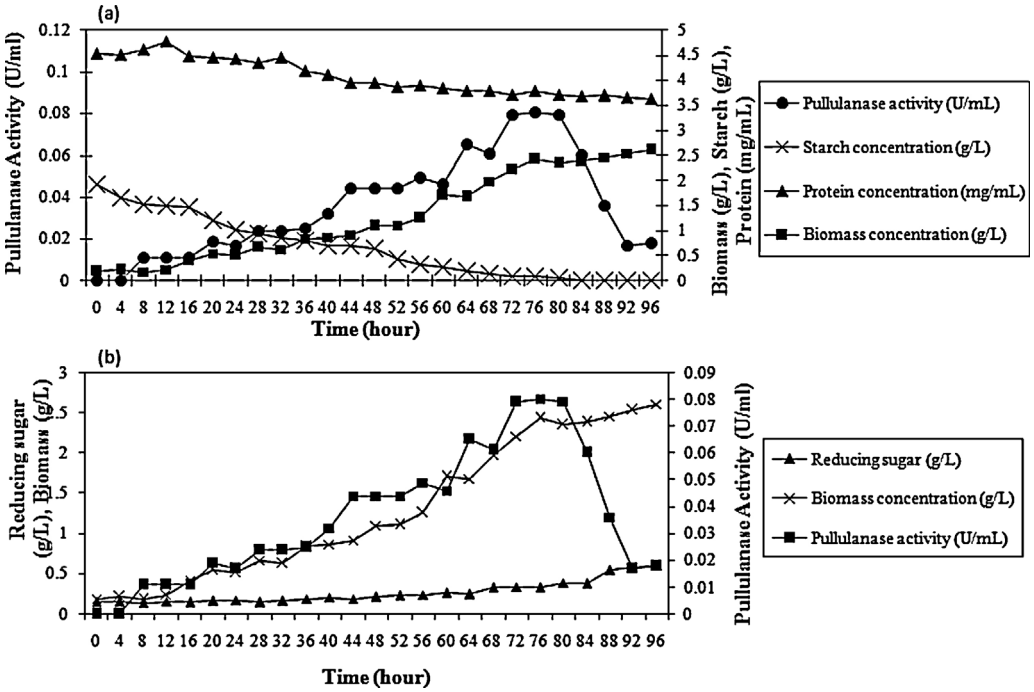

Fig. 2. Time course of batch culture for pullulanase production by *Bacillus flavothermus* KWF-1. (a) Effect of starch concentration on pullulanase activity. (b) Effect of reducing sugar production on pullulanase activity.

Table 1
Effect of fix mode on pullulanase production.

Analysis	Result
Max pullulanase activity (U/mL)	0.066 ± 0.005
Protein (mg/mL)	3.387
Max specific activity (U/mg)	0.0195
X_{max}	1.13 ± 0.06
t to reach max pullulanase (h)	24
Max reducing sugar (g/L)	1.004 ± 0.018
Final starch concentration (g/L)	0.961

Similar observation was also reported by several researchers who accounted that pullulanase from *Klebsiella* sp. is initially localized to the outer membrane and released into the medium when the cells reached exponential phase (Michaelis, Chapon, D'enfert, Pugsley, & Schwartz, 1985; Murooka & Ikeda, 1989).

Fig. 2b indicates that reducing sugar concentration increases parallel with pullulanase activity (Fig. 2a). At the end of cultivation, 0.607 g/L of reducing sugar was detected in the culture. Pullulanase activity found to decrease rapidly at the late stationary phase where reducing sugar concentration was at peak. This may be due to increase of reducing sugar concentration that inhibits the production of pullulanase. Increase in biomass concentration resulted in rapid utilization of starch. Starch hydrolysis contributes to increase of reducing sugar concentration due to action of amylolytic enzymes such as pullulanase and α-amylase secreted by the bacteria to produce simple sugars such as glucose, maltose and maltotriose (Bertoldo and Antranikian, 2002). Subash Nair et al. (2006) stated that pullulanase activity produced by *Bacillus cereus* FDTA-13 dropped in the presence of high glucose concentration in the medium (8.0 $mmol^{-1}$).

3.3. Production of pullulanase in fed batch culture

3.3.1. Effect of fix mode on pullulanase production

Fix mode feeding was unable to enhance pullulanase production compared to batch culture. Maximum pullulanase activity of 0.066 U/mL was attained after 24 h of incubation. Pullulanase production was 1.22-fold lower than the batch culture. The maximum biomass concentration of 1.13 g/L and pullulanase specific activity of 0.0195 U/mg obtained was respectively 2.3 and 1.09-fold lower than in the batch culture. However, reducing sugar concentration of 1.004 g/L in the medium was 1.7-fold higher than in the batch culture (Table 1). Decrease in pullulanase activity may due to high concentration of unutilized sugar in the medium. Hii et al. (2009b) suggested that increase of pullulanase activity can be attained when reducing sugar in the medium is instantly consumed by the culture. The presence of reducing sugars may repress the production of pullulanase (Brandt, Catley, & Awad, 1976).

3.3.2. Effect of variable volume constant mode on pullulanase production

In constant feed rate (*F*) fed-batch system, the feeding rate was kept constant from the beginning of experiment. In this operation, dilution rates changes as volume in vessel increased with time. Screening for suitable feeding rate was performed by investigating different dilution rates for improved pullulanase production. The constant feeding mode was operated at dilution rates (*D*) of 0.005, 0.01, 0.03 and 0.05 h^{-1}. Application of constant feed rate mode was found to significantly improve pullulanase production compared to fix mode and batch culture. Maximum pullulanase activity of 0.0618 U/mL, 0.1051 U/mL, 0.1016 U/mL and 0.0215 U/mL were observed at dilution rates of 0.005, 0.01, 0.03 and 0.05 h^{-1} respectively (Table 2). The highest increment of pullulanase production by 1.31-fold compared to batch culture and 1.59-fold compared to fix mode fed batch culture was achieved at (μ) 0.01 h^{-1}. Increase of dilution rate to 0.03 h^{-1} and 0.05 h^{-1} resulted in lower pullulanase activity. This reduction may be due to substrate inhibition as increase of feed rate increases the accumulation of unutilized sago starch. On the other hand, this experiment shows that at lower dilution rate, decrease in pullulanase activity was also observed. At μ = 0.005 h^{-1}, pullulanase activity was 1.30-fold lower than the batch culture (0.0618 U/mL). Complete depletion of starch at the end of fermentation was observed. This observation may be due to insufficient nutrient supply at lower feeding rate which leads to cell death and decreases in pullulanase activity.

High specific activity of 0.0491 U/mg was obtained at μ = 0.01 h^{-1}, which was 2.31-fold higher than in batch culture. The maximum value of biomass concentration (X_{max}) at all dilution rates in constant fed batch culture was lower compared to batch culture. This may be due to incompatible feeding mode which was unable to provide appropriate amount of starch for biomass production. At lower dilution rate, insufficient substrate supply may lead to reduction of cell growth. Inversely, higher dilution rate may lead to biomass dilution. Shene, Andrews, and Asenjo (1999) stated that decrease in biomass yield may occur due to dilution of biomass when culture volume increased with time.

3.3.3. Effect of variable volume exponential mode on pullulanase production

Exponential feeding strategy greatly improved pullulanase production compared to batch culture, fix mode and variable constant mode. As shown in Fig. 3a, feeding rate was increased stepwise every 8 h to obtain an exponential increase of biomass. An improvement of 2.13-fold of pullulanase activity (0.171 U/mL) was achieved

Table 2
Effect of various dilution rates in constant feeding mode.

Feeding mode	Batch	Variable mode-constant (/h)			
Flow rate (/h)		0.05	0.03	0.01	0.005
Max pullulanase activity (U/mL)	0.0803[a]	0.0215[a]	0.1016[b]	0.1051[b]	0.0618[a]
Protein (mg/mL)	3.77 ± 0.113	2.203 ± 0.111	2.462 ± 0.117	2.141 ± 0.113	3.061 ± 0.112
Max specific activity (U/mg)	0.0213	0.0098	0.0413	0.0491	0.0202
X_{max}	2.61 ± 0.134	1.27 ± 0.128	1.67 ± 0.125	2.15 ± 0.127	1.37 ± 0.127
t to reach max pullulanase (h)	76	8	40	64	4
Max reducing sugar (g/L)	0.607[c]	0.336[c]	0.638[d]	0.857[e]	1.421[d]
Final starch concentration (g/L)	0.001[b]	3.5 ± 0.118	1.9 ± 0.121	1.3 ± 0.114	0.001[a]

Sd:
[a] ±0.003.
[b] ±0.002.
[c] ±0.014.
[d] ±0.013.
[e] ±0.011.

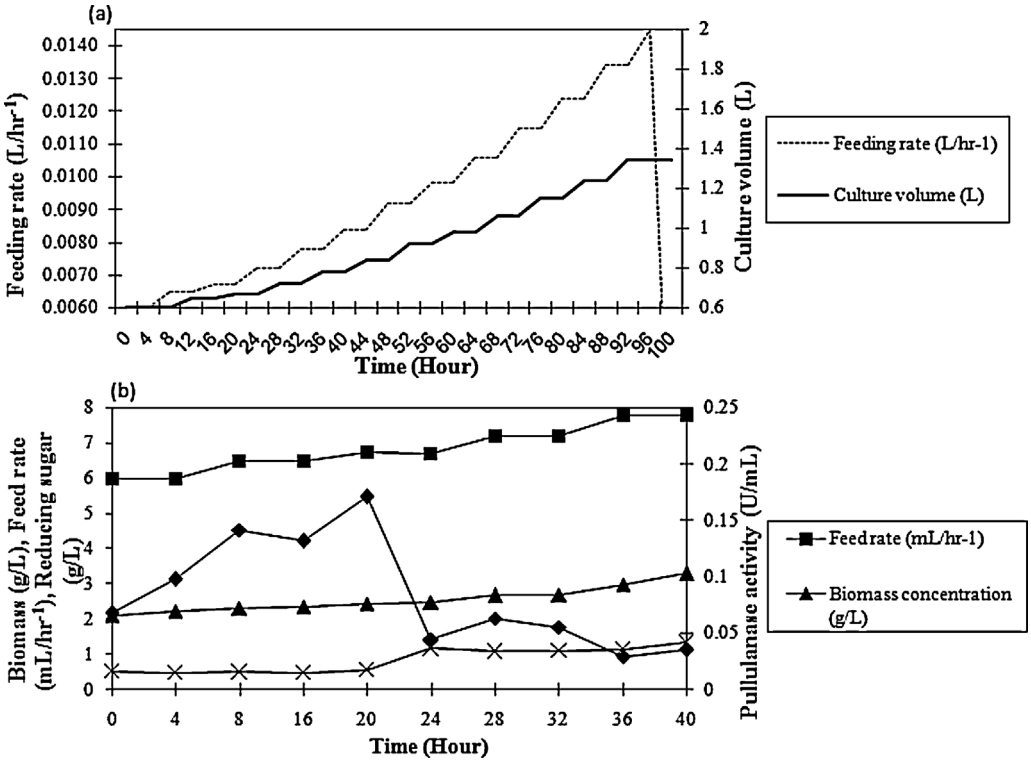

Fig. 3. Exponential fed batch culture for pullulanase production by *Bacillus flavothermus* KWF-1. (a) Feed rate profile for stepwise exponential fed batch fermentation ($\mu = 0.01\ h^{-1}$). (b) Time course of exponential fed batch culture.

compared to batch culture using exponential feeding method with dilution rate of 0.01 h^{-1}. However, increase of dilution rate to 0.03 h^{-1} failed to improve pullulanase activity (0.0761 U/mL). This observation was almost similar with the result obtained by Kim and Hou (2006). They reported that the production of lipase by *Candida cylindracea* in exponential fed batch culture decreased when dilution rate was increased. At lower dilution rate (0.005 h^{-1}), pullulanase activity attained was only 1.29-fold higher than batch culture (0.1032 U/mL). This may be due to insufficient supply of substrate to the culture whereby complete depletion of starch was observed at the end of the fermentation as shown in Table 3. Maximum pullulanase specific activity of 0.0657 U/mg was obtained at dilution rate of 0.01 h^{-1}, which was 3.09-fold higher than batch culture. In variable volume exponential fed batch culture, formation of an ideal culture condition with increase of growth limiting substrate results in exponential growth of microorganism. In Fig. 3b, slow increment of biomass concentration can be observed as the feed rate increased with time. Maximum biomass concentration of 3.3 g/L, which was 1.26-fold higher than in batch culture was obtained.

3.4. Effect of sago starch concentration in variable volume exponential mode feeding on pullulanase production

A series of experiments were conducted to screen the effect of sago starch concentration on pullulanase production in the feeding medium. Screening was performed by feeding the culture with sago starch at concentrations of 1% (w/v), 2% (w/v) and 3% (w/v) using exponential feeding method with dilution rate of 0.01 h^{-1}. Table 4 summarizes the effect of sago starch concentration on pullulanase production. Feeding with 1% (w/v) sago starch resulted in increment of pullulanase activity of 0.1449 U/mL, which was 1.80-fold higher than batch culture. Biomass concentration was 1.2-fold lower than batch culture with 2.13 g/L. This may be due

Table 3
Effect of various dilution rates in exponential feeding mode.

Feeding mode	Batch	Variable mode-exponential (/h)		
Flow rate (/h)		0.03	0.01	0.005
Max pullulanase activity (U/mL)	0.0803[a]	0.0761[a]	0.171[a]	0.1032[c]
Protein (mg/mL)	3.77 ± 0.113	2.563 ± 0.120	2.603 ± 0.131	2.966 ± 0.118
Max specific activity (U/mg)	0.0213	0.0297	0.0657	0.0348
X_{max}	2.61 ± 0.134	2.97 ± 0.128	3.3 ± 0.131	3.12 ± 0.127
t to reach max pullulanase (h)	76	24	20	32
Max reducing sugar (g/L)	0.607[d]	0.421[e]	1.936[f]	0.737[e]
Final starch concentration (g/L)	0.001 ± 0.002	1.0 ± 0.119	0.5 ± 0.123	0.002[b]

Sd:
[a] ±0.003.
[b] ±0.004.
[c] ±0.005.
[d] ±0.014.
[e] ±0.016.
[f] ±0.018.

Table 4
Effect of sago starch concentration on pullulanase production.

Carbon source concentration (w/v)	Exponential Feeding 0.01 (/h)		
	1%	2%	3%
Max pullulanase activity (U/mL)	0.1449 ± 0.010	0.1709 ± 0.008	0.0987 ± 0.006
Protein (mg/mL)	2.882 ± 0.117	2.603 ± 0.124	3.146 ± 0.118
Max specific activity (U/mg)	0.0503	0.066	0.0314
X_{max}	2.13 ± 0.126	3.3 ± 0.112	2.3 ± 0.119
t to reach max pullulanase (h)	24	20	16
Max reducing sugar (g/L)	0.564 ± 0.012	1.936 ± 0.014	0.32 ± 0.019
Final starch concentration (g/L)	0.1 ± 0.111	0.5 ± 0.113	3.5 ± 0.142

to diluted starch concentration compared to batch culture. However, increase of starch concentration in feeding medium improved biomass and pullulanase production. Feeding with 2% (w/v) of sago starch concentration gave the highest pullulanase activity of 0.171 U/mL, which was 2.13-fold higher than batch culture. Feeding with sago starch concentration at 2% (w/v) also resulted in highest biomass concentration of 3.3 g/L, which was 1.26 higher than in batch culture. This result was similar to the result reported by Singh, Vohra, and Sahoo (2004) in protease production by *Bacillus sphaericus* where the experiment showed that biomass concentration rose when carbon concentration was increased. Increase of starch concentration in the medium would increase the pullulanase action on sago starch (with α-[1,6]-linkages) thus will increase the pullulanase activity. It was supported by Subash Nair et al. (2006) that claims presence of α-(1,6)-linkages in complex polysaccharides can induce the production of pullulanase.

Increase to 3% (w/v) of sago starch concentration resulted in lower pullulanase activity (0.0987 U/mL) compared to feeding with 2% (w/v) sago starch. As shown in Table 4, final starch residue of 3.5 g/L in the fermentation medium suggests that substrate inhibition may have occurred due to high unutilized starch concentration. Liaw et al. (2001) suggest that decrease of pullulanase activity when substrate concentration increased may be due to substrate inhibition or limitation of other essential nutrients. This was similar to the study performed by Long, Huang, Li, and Ye (2008) who reported that activity of epoxysuccinate hydrolase decreased when substrate concentration was increased. Another similar observation was obtained in the research by Singh et al. (2004), which is the increased production of protease in fed batch culture. According to the report, protease activity decreased when concentration of glucose as carbon source increased.

3.5. Production of maltose, maltotriose and panose in batch and exponential fed batch culture

Effect of different fermentation systems on various polyoses production was studied. Fed batch system proven to improve production of both maltotriose and panose. Table 5 presents the concentration of different sugars in batch and fed batch culture. Increase of maltotriose concentration (1.046 g/L) in exponential fed batch culture up to 4.3-fold compared to batch culture (0.243) was observed. In a reported research by Singh et al. (2010a, 2010b), 4.2 g/L of maltoriose was obtained using purified pullulanase from *Bacillus acidopullulyticus* and pullulan as substrate. Higher concentration of maltotriose attained may be due to pullulan specific properties where higher amount of α-1,6 linkage present in pullulan compared to sago starch for hydrolyzation. Similarly, concentration of panose increased 2.5-fold in fed batch culture at 0.277 g/L compared to batch culture at 0.112 g/L. Similar observation was reported by Fernandes and Rodrigues (2006) who based their research on panose production using sucrose as substrate and dextran-saccharase enzyme from *Leuconostoc mesenteroids* NRRL B512F. Based on their research, Panose productivity in fed batch culture was found to be 74% higher than batch culture due to long period of higher reaction rate. On the other hand, concentration of maltose of 0.101 g/L in fed batch culture was 1.1-fold lower than batch culture. This may be due to high pullulanase production in fed batch culture that capable of breaking α-1,6 linkage which further leads to assimilation of maltose (Fernandes & Rodrigues, 2006; Hang & Woodams, 1995; Rodrigues, Lona, & Franco, 2006; Yun, Lee, & Song, 1994).

Table 5
Effect fermentation technique on different polyoses production.

Sugar (g/L)	Batch	Fed batch
Maltose	0.113 ± 0.012	0.101 ± 0.014
Maltotriose	0.243 ± 0.013	1.046 ± 0.012
Panose	0.112 ± 0.012	0.277 ± 0.015

4. Conclusions

Application of congo red in qualitative detection of pullulanase activity on pullulan agar plate notably increased the distinction between the clearing zone and the background. Based on this study, fed batch culture was proven to significantly increase the pullulanase production compared to batch culture. Exponential feeding strategy produce the highest pullulanase activity compared to other feeding modes. Maximum pullulanase production of 0.171 U/mL, which was 2.13-fold higher compared to batch culture, was obtained using 2% (w/v) of sago starch and exponential feeding mode at dilution rate of 0.01 h^{-1}. Sago starch was successfully utilized as limiting substrate for the production of pullulanase. This study can be effectively applied in industries to obtain continuous high pullulanase production more economically. Fed batch system was also proven to increase the production of polyoses from hydrolyzation of sago starch. Maltotriose and panose are two important types of sugars that have high commercialization value. Thus, improvement in the production through fed batch system based on this research will aid the food industry to improve their production.

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