Enhancement of Extracellular Pullulanase Production by *Raoultella planticola* DSMZ 4617 Using Optimized Medium Based on Sago Starch

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Abstract: Enhancement of pullulanase production by *Raoultella planticola* DSMZ 4617 using optimized medium formulation was investigated in batch fermentation using 500-mL shake flask. The fermentations were carried out, firstly, to search for a suitable cultivation medium for enzyme production and followed by the evaluations on the influence of carbon and nitrogen sources and also initial culture pHs on the secretion of pullulanase by this bacterium. The modified mineral Czapek medium was found suitable to produce substantially high activity of pullulanase (320 times higher) as compared to 'Ohba-Ueda' medium. This bacterium was found superior in pullulanase production using sago starch and peptone as carbon and nitrogen sources, respectively. Using the optimized medium, the bacterium produced 0.95 U/mL of pullulanase at initial culture pH of 7 and incubation temperature of 30°C.

Key Words: Pullulanase, *Raoultella planticola* DSMZ 4617, submerged fermentation, sago starch.

INTRODUCTION

Pullulanase (EC 3.2.1.41) is a direct debranching enzyme that catalyzes the hydrolysis of α-1,6-glucosidic bonds of unmodified substrate, for example, amylopectin and/or glycogen and related polymers. The main function of pullulanase was to improve the efficiency of starch saccharification process. It is because pullulanase would specifically hydrolyze the branch points in the amylopectin residues whereas the glucoamylase has only to hydrolyze the linear 1,4-α-glucosidic linkages when pullulanase and glucoamylase are simultaneously used during saccharification process. As a result, the maximum dextrose levels achieved are higher [1]. Therefore, the use of a debranching enzyme would speed the overall saccharification process and reduce the total amount of glucoamylase that is required for complete conversion process. The practical advantage of using pullulanase together with glucoamylase is that less glucoamylase activity would be used. This will allow a reduction in the use of glucoamylase up to 60% and therefore less enzyme catalyzed polymerization of D-glucose to isomaltose takes place [2].

Varieties of carbon-containing substrates have been used as carbon source for pullulanase fermentation by microorganisms, for example, pullulan [3], maltose [3, 4] and starch [5, 6]. Overall, the use of starch as a carbon source was found to yield the pullulanase which is intermediate to high in comparison to other carbon sources. The rate of pullulanase synthesis was also appeared to vary with the nature of nitrogen source and most of the studies on the influence of nitrogen source on pullulanase production were performed using organic nitrogen sources such as yeast extract, peptone, tryptone, trypticase, casamino acids and corn steep liquor [5, 7, 8].

Pullulanase fermentation by gram positive bacteria has been well-reported and commercial varieties of pullulanase are mainly derived from either *Bacillus acidopullulyticus* or *Klebsiella planticola* [9] or *Bacillus deramificans* (expressed in *Bacillus licheniformis*) [10]. However, information on pullulanase synthesis by a gram negative bacterium, such as, *Raoultella planticola*, which has been reported as superior pullulanase producer, is very limited. The present study was undertaken to investigate the nutrients requirements and cultural conditions for enhancement of pullulanase production from this bacterium. Preliminary, selection of the suitable medium formulation for pullulanase fermentation by *R. planticola* was conducted. Subsequently, the feasibility of using different types of starch as carbon source for the production of pullulanase was carried out. Influence of different types of nitrogen source and initial culture pH were also investigated.

MATERIALS AND METHODS

Microorganism and Inoculum Preparation

The pullulanase-producing bacterium, *Raoultella planticola* DSMZ 4617, was used in this study. The strain was stored at -80°C in 30% (v/v) glycerol. For inoculum preparation, five loops of culture from the stock was sub-cultured

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into the red pullulan agar (Megazyme, Ireland). A single colony from the red pullulan agar was inoculated into 500 mL Erlenmeyer flask containing nutrient broth (Merck, Germany) and incubated at 30°C for 24 h to obtain an initial cell concentration with optical density of around 0.5 read at 550 nm. This culture was used for inoculum (10% v/v) for all fermentations carried out in this study.

Selection of Cultivation Medium

Brandt *et al*. [11] found that extracellular pullulanase by a *Klebsiella* sp. was obtained in a modified mineral Czapek medium with maltose as a carbon source. On the other hand, Ohba and Ueda [12] reported that pullulanase from *Aerobacter aerogenes* RS-1 was produced in a medium similar to the modified mineral Czapek medium with potato starch as a carbon source and ammonium acetate as a nitrogen source. The feasibilities of using modified mineral Czapek and 'Ohba-Ueda' media were investigated in this study and the media were prepared according to the methods as described by Brandt *et al.* [11] and Ohba and Ueda [12], respectively (Table **1**). Experiment to study the effect of different carbon sources was carried out by replacing the carbon sources, according to the need of each experiment. Gelatinized starches were prepared by heating starch slurry to slightly above 70°C. The effect of various organic nitrogen sources, i.e., meat extract, tryptone, tryptose, yeast extract, bacteriological peptone and peptone (Difco Laboratories, USA) was studied with sodium nitrate as the inorganic nitrogen source. Subsequently, the effect of different inorganic nitrogen sources (ammonium sulfate and ammonium acetate – Merck, Germany) on pullulanase production was performed by replacing sodium nitrate and peptone was used as an organic nitrogen source. Unless stated elsewhere, all replacements were performed with the same concentration of each nitrogen source.

Table 1. Composition of the Selected Growth and Production Media

Modified Mineral Czapek Medium (Brandt *et al*., 1976)		'Ohba-Ueda' Medium (Ohba and Ueda, 1982)	
Compound	**(% w/v)**	**Compound**	**(% w/v)**
K_2HPO_4	0.1	CH_3COONH_4	0.8
$NaNO_3$	0.5	K_2HPO_4	0.2
$MgSO_4.7H_2O$	0.05	$MgSO_4.7H_2O$	0.075
KCl	0.05	$FeSO_4.7H_2O$	0.005
$FeSO_4.7H_2O$	0.001	Peptone	0.03
Peptone	0.8	Potato starch	1.5
Maltose	0.5		

* Source of chemicals: K_2HPO_4, KCl (Univar Ajar Chemical, Australia); $NaNO_3$, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, CH_3COONH_4 (Merck, Germany); maltose (Sigma Aldrich, USA), potato starch (local hypermarket, Malaysia)

Submerged Fermentation

The production of pullulanase was carried out as submerged fermentation using 500-mL Erlenmeyer flask containing 200 mL of liquid medium. In all experiments, initial pH of medium was adjusted to 7.0 using 1N HCl, except for the study on the effect of initial culture pH on growth and pullulanase production where the initial pH of medium was adjusted to the required value prior to sterilization. The flasks were incubated at 30°C and agitated at 250 rpm in an orbital shaker (B. Braun Biotech International, Germany). All fermentations were carried out in triplicate and the results presented were average values.

Analytical Procedures

Pullulanase activity was determined by measuring the enzymatic release of reducing sugars during incubation with pullulan (from *Aureobasidium pullulans*; Sigma-Aldrich Chemie Gmbh). The reaction mixture contained 1% pullulan in 0.02 M sodium phosphate buffer, pH 6.9 and a sample of the enzyme, in a final volume of 1 mL. After incubation at respective temperatures for 30 min, concentration of reducing sugar was determined using 3, 5-dinitrosalicylic acid method where the absorbance was read at 540 nm [13]. One unit of pullulanase activity is defined as the amount of enzyme required to produce 1 µmol reducing sugar (equivalent to maltotriose) per minute under the assay conditions.

Concentration of starch was determined colorimetrically [14] on the basis of iodine starch complex color with some modification. Supernatant from a culture broth was mixed with 0.1 M phosphate buffer (pH 7.2, 60 µL), 5 M NaCl (200 µL), 1 M HCl (40 µL), iodine reagent (50 µL) and distilled water (10 mL). The absorbance for the mixture was then read at 625 nm.

Dry cell weight was determined by filtration and oven drying method. Samples (after treatment with α-amylase, 0.2 mL) were filtered through a pre-weighed 0.2 µm cellulose nitrate membrane filter (Whatman) by using a vacuum pump. The cells were then dried in an oven at 95°C until a constant weight was achieved.

RESULTS AND DISCUSSION

Selection of Cultivation Medium for Growth and Pullulanase Production

The performance of pullulanase production by *R. planticola* DSMZ 4617 using different cultivation media (modified mineral Czapek medium and 'Ohba-Ueda' medium) is summarized in Table **2**. Pullulanase production by *R. planticola* DSMZ 4617 in modified mineral Czapek medium (0.32 U/mL) was about 320 times higher than that obtained in fermentation using 'Ohba-Ueda' medium (0.001 U/mL).

Pullulanase yield was much better (137.8 unit of enzyme produced per unit of substrate utilized) when the fermentation was conducted using modified mineral Czapek medium than 'Ohba-Ueda' medium. The stimulatory effect of maltose on the induction of pullulanase production by microorganism has been reported and discussed in several past researches. According to Brandt *et al*. [11], synthesis of pullulanase can be enhanced by pullulan, maltotriose and maltose. Gomes *et al*. [15] also proved that among the various carbon sources tested, maltose was the most effective for the production of pullulanase by *Rhodothermus* marinus. Moreover, several studies reported that the production of pullulanase, in both *Klebsiella pneumoniae* and *E. coli* K-12 carrying the cloned *pulA* gene, was stimulated by growth in the presence

of maltose and positively regulated by MalT protein, activator of the maltose regulon [3, 16]. Hence, the pullulanase was overproduced and secreted into the culture medium.

Growth measured as cell concentration under 'Ohba-Ueda' medium was much lower (0.26 g/L) than fermentation using modified mineral Czapek medium (0.72 g/L). The presence of high concentration of organic nitrogen source, i.e., 0.8 g/L of peptone, in modified mineral Czapek medium as compared to 0.03 g/L peptone in 'Ohba-Ueda' medium might play an important role in cell growth and pullulanase synthesis inside the cell before it is secreted into the culture medium [17]. Suzuki and Chishiro [18] reported that pullulanase synthesis by *B. steorothermophilus* KP 1064 was stimulated by organic nitrogen sources. Medium containing only ammonium sulfate or ammonium acetate did not enhance amylases production by *B. cereus* BQ 10-S1 Spo [19]. Since the production of pullulanase by *R. planticola* DSMZ 4617 in modified mineral Czapek medium was significantly higher as compared to 'Ohba-Ueda' medium, this medium was chosen and applied in the subsequent experiments.

Production of Pullulanase Using Different Types of Starch

The time-course of pullulanase fermentation by *R. planticola* DSMZ 4617 using starch as carbon source is given in Fig. **(1A-C)**. The bacterium grew well in all types of starch used except for corn-based medium where the cell concentration was significantly lower than that in other starch-containing media. Growth was rapid (specific growth rate = 0.17 h^{-1}) during the initial stages of the fermentation (10 to 16 h of cultivation) and reached a stationary phase after 48 h, except for corn starch as cell growth ceased before level off (Fig. **1B**). Marlida *et al.* [20] reported that due to starch composition (less amylopectin and high amylose content), corn starch was more resistant to enzyme action and therefore required much longer hydrolysis time before it can be 'consumed' by the microorganisms for biomass built-up.

The highest extracellular pullulanase production was found in fermentation using sago starch as the sole carbon source, with maximum pullulanase activity of approximately 0.95 U/mL, followed by potato (0.85 U/mL), tapioca (0.68 U/mL) and corn (0.14 U/mL) (Table **3**). In all cases, pullulanase activity has not been detected during the early stages of fermentation (0-24 h) (Fig. **1A**). Active enzyme production was only started during the later stages of exponential growth phase. Similar observations were also reported by many researchers who stated that pullulanase from *Klebsiella* sp. is initially localized to the outer membrane and is released into the medium when the cells have completed exponential growth [3, 21]. In addition, the lowest production of pullulanase enzyme in corn-based medium (0.14 U/mL) may be related to the lowest cell production (0.38 g/L). The results obtained indicated that sago starch-based medium could be used as a favorable cultivation medium for the growth of culture and also enzyme production by *R. planticola* DSMZ 4617.

Among the four types of starch investigated, the highest yield (182 unit pullulanase/g starch) and productivity (0.0079 U/mL·h) was obtained when sago starch was used (Table **3**). The variations in pullulanase production by *R. planticola* DSMZ 4617 in medium containing different types of starch might be due to the fact that starches from different sources differ in overall structure through size distribution of the granules, shape, amylose and lipid content, distribution of chain length in amylopectin and crystalline structure [22]. Moreover, lower degree of intermolecular association in sago starch granules might cause it to be easily hydrolyzed by enzymes [23], such as pullulanase. Protein content in sago starch is very low but is exceptionally high in soluble carbohydrates, therefore, sago starch could be as effective as other commercial starches used in fermentation industry [24].

From Fig. (**1C**), the estimated volume of starch slurry in the culture system containing potato, tapioca and sago starches decreased almost linearly after the pullulanase had been detected in the culture fluid and also outer surface of the cells. After 48 h of fermentation, there was no significant difference among the residual concentration of potato (0.3 g/L), tapioca (0.1 g/L) and sago (0.1 g/L) starches. The residual concentration of corn (4.0 g/L) was significantly different from the others in which almost 70% of starch remained unconsumed even after 144 h of fermentation.

The maximum rate of starch hydrolysis ($-dS/dt_{max}$), calculated by measuring the maximum tangent gradient of the curves when starch concentration was plotted against fermentation time, varied with the type of starches employed. The $-dS/dt_{max}$ was approximately 0.191 g/L/h for potato starch, 0.237 g/L/h for tapioca starch, 0.208 g/L/h for sago starch and 0.032 g/L/h for corn starch, respectively. It is interesting to note that the reducing sugars were not detected in culture during the fermentation period, suggesting that the uptake of reducing sugars was faster than the release of residual reducing sugar from starch hydrolysis. This was one of the advantages of using starch as the carbon source for pullulanase production. The presence of glucose and other readily metabolizable sugars might repress pullulanase synthesis [11].

During the initial stages of fermentation (0-24 h), the culture pH was increased from pH 7 to pH 9. The pH was slightly decreased when the cells started to release pullulanase onto the culture and at the same time the starch was hydrolyzed, indicating that the starch hydrolysis could be one of the hydrogen-producing processes [2]. Then, the culture pH started to increase to around pH 8.5 after cell growth and production of extracellular pullulanase reached maximum levels.

This study clearly demonstrated that *R. planticola* DSMZ 4617 was a suitable microorganism for the production of pullulanase by using gelatinized sago starch as a carbon source. It has high ability to produce pullulanase enzyme during growth on sago starch as compared to maltose which has not been reported elsewhere. This is an attraction in reducing the overall production cost since sago starch is normally classified as cheap source of carbon (USD 0.5 per kg as supplied by Song Ngeng Sago Ind. (EM) Sdn Bhd, Malaysia) compared to maltose (USD 226 per kg as supplied by Sigma-Aldrich, USA). Furthermore, sago starch which is abundantly available in Malaysia [24], is an interesting alternative carbon source for pullulanase fermentation by this bacterium. During the fermentation process, *R. planticola* DSMZ 4617 offered an advantage of no protease being released (see Table **3**) which implicates the versatile usage of

this microorganism as pullulanase-producer with better enzyme stability.

Nitrogen Requirements

The time courses of pullulanase fermentation by *R. planticola* DSMZ 4617 using different types of organic and inorganic nitrogen sources are shown in Fig. (**2**) and the performance of each fermentation are summarized in Tables **4** and **5**. The organic nitrogen source used greatly influenced both growth and pullulanase production (Table **4**). For all types of organic nitrogen investigated, the fermentation time to reach maximum pullulanase activity was more than 120 h (Fig. **2A**). Except meat extract and bacteriological peptone, all organic nitrogen sources tested were found to be capable of producing pullulanase enzyme extracellularly with activity more than 0.3 U/mL. The highest pullulanase activity (0.95 U/mL) was detected in medium containing peptone, followed by tryptone, yeast extract, tryptose, meat extract and bacteriological peptone with the activity of 0.87 U/mL, 0.40 U/mL, 0.36 U/mL, 0.24 U/mL and 0.18 U/mL, respectively.

The yield of pullulanase produced based on utilized starch ($Y_{P/S}$) and productivity (P) were significantly different for different types of organic nitrogen sources used. The highest yield ($Y_{P/S}$) (182 U enzyme g starch^{-1}) and maximum productivity (P) (0.0079 U/mL·h) was obtained in fermentation using peptone. Type of organic nitrogen source also greatly influenced the rate of hydrolysis of starch during cell growth and pullulanase production phase. Starch was hydrolyzed effectively in medium supplemented with tryptone or peptone as compared to other nitrogen sources (Fig. **2B**). More than 40% of starch supplied still remained in the culture for fermentation using meat extract and bacteriological peptone even after 120 h of cultivation.

In order to investigate whether the culture produce the enzyme effectively or the production of enzyme is simply proportional to cell mass, the maximum activity of pullulanase was calculated based on a unit of cell weight (P_{max}/X_{max}) [25]. From the results obtained (Table **4**), all tested organic nitrogen sources could promote the growth of the culture (X_{max} = 0.55 g/L to 0.70 g/L). The ability of the culture to produce pullulanase, however, decreased in medium containing bacteriological peptone, meat extract, yeast extract and tryptose with the ratio of P_{max}/X_{max} in the range of 335 unit/g to 576 unit/g. The highest value of P_{max}/X_{max} was achieved in medium containing peptone (1484 unit pullulanase/g cell) and thus indicated that peptone could be one of the favorable organic nitrogen sources that enhance the cell growth of the culture as well as the pullulanase production.

Table 2. Effects of Two Different Cultivation Media (Modified Mineral Czapek Medium and 'Ohba-Ueda' Medium) on Pullulanase Production by *R. planticola* DSMZ 4617 in Shake Flask Fermentation

Parameters	Medium	
	Modified Mineral Czapek	'Ohba-Ueda'
P_{max} (U/mL)[1]	0.32[a]	0.001[b]
t_p (h)	36[b]	43[a]
X_{max} (g/L)[1]	0.72[a]	0.26[b]
t_x (h)	36[b]	42[a]
S (x 10^{-3} g/mL)	2.36[a]	1.48[b]
P (x 10^{-3} U/mL·h)	9.00[a]	0.05[b]
$Y_{P/S}$ (U pullulanase/g starch)	137.8[a]	0.7[b]

[1] Maximum value of pullulanase activity and cell concentrations were the highest values attained during cultivation.
[a-b] Mean values in the same row not followed by the same letter are significantly different (P<0.05).

Among the three inorganic nitrogen compounds used, sodium nitrate and ammonium sulfate were found to be better than ammonium acetate for production of pullulanase by *R. planticola* DSMZ 4617 (Table **5**). Sodium nitrate and ammonium sulfate highly support good growth of *R. planticola* DSMZ 4617 than ammonium acetate.

Table 3. Comparison of the Performance and Kinetic Parameter Value of Pullulanase Fermentation by *R. planticola* DSMZ 4617 in Shake Flask Using Different Types of Starch

Parameters	Starch			
	Potato	Tapioca	Corn	Sago
P_{max} (U/mL)	0.85[b]	0.67[c]	0.14[d]	0.95[a]
t_p (h)	144[a]	120[b]	72[c]	120[b]
X_{max} (g/L)	0.62[a]	0.64[a]	0.38[b]	0.64[a]
t_x (h)	72[b]	96[a]	96[a]	72[b]
S (x 10^{-3} g/mL)	4.90[c]	5.10[b]	1.48[d]	5.23[a]
P (x 10^{-3} U/mL·h)	5.93[b]	5.60[c]	1.96[d]	7.92[a]
$Y_{P/S}$ (U pullulanase/g starch)	174.1[b]	131.6[c]	95.6[d]	181.6[a]
Maximum protease activity (μg/mL)	N.D.[1]	N.D.	N.D.	N.D.

[a-d] Mean values in the same row not followed by the same letter are significantly different (P<0.05).
[1] N. D. - Not Detectable.

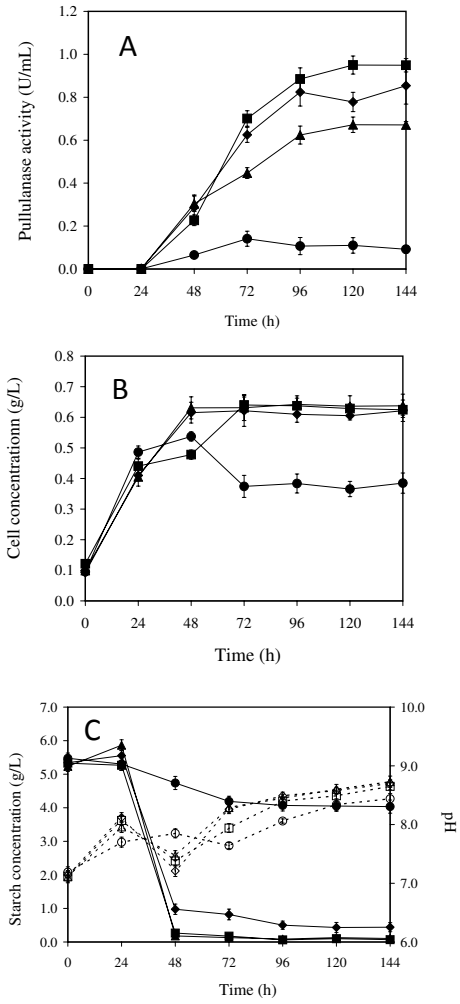

Fig. (1). Time course of pullulanase production by *R. planticola* DSMZ 4617 using starch as carbon source. (**A**), pullulanase activity; (**B**), growth profile; (**C**) starch concentration (solid lines) and pH (dotted lines). Symbols represent: (◆), potato starch; (▲), tapioca starch; (●), corn starch; (■), sago starch. Error bars indicate the mean ± standard deviation of triplicate.

The differences in the amount of amino acids content and/or type of amino acid present in different nitrogen sources might be one of the reasons why production of enzymes by microorganisms can be repressed or activated [26]. For example, Antranikian *et al.* [17] found that with the presence of organic nitrogen source in the cultivation medium, more than 90% of the enzyme was released into the culture fluid by *Clostridium thermohydrosulfuricum* DSM 567. Swamy and Seenayya [5] also reported that among the different nitrogen sources tested, peptone was found to be more preferred for extracellular and cell bound pullulanase production by *Clostridium thermosulfurogenes* SV9.

Effect of Initial Culture pH

The time courses of pullulanase fermentation by *R. planticola* DSMZ 4617 at different initial culture pH values are shown in Fig. (**3**) and the performance of each fermentation is given in Table **6**. Different initial culture pH values greatly influenced the extracellular pullulanase production by *R. planticola* DSMZ 4617 (Fig. **3A**). At initial culture pH between 5 to 8, there was significant difference on extracellular pullulanase production, where the maximum activity detected was approximately 0.5 U/mL, 0.8 U/mL, 1.0 U/mL and 0.6 U/mL for pH 5, pH 6, pH 7 and pH 8, respectively. Thus, *R. planticola* DSMZ 4617 was found to be able to produce extracellular pullulanase at the initial pH of 6 to 8. Ohba and Ueda [12] reported that the optimal range of initial pH for extracellular pullulanase production by *Aerobacter aerogenes* RS-1 was between pH 7 to pH 9. dos-Santos *et al.* [27] also reported that the minimum initial culture pH to be set at 6 prior to the fermentation process, indicating that the *Klebsiella oxytoca* P2 under study performed well in that pH value.

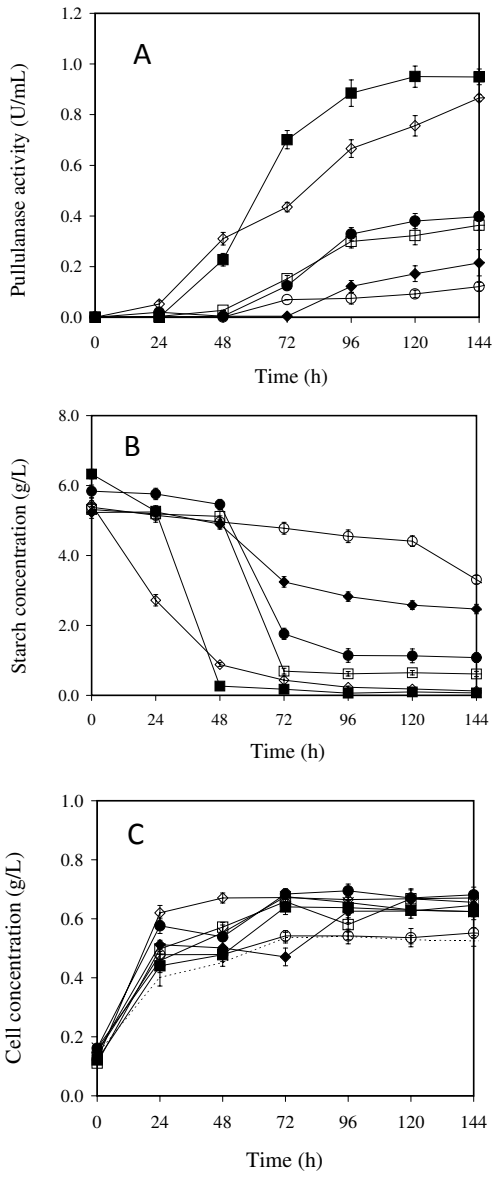

Fig. (2). Time course of pullulanase production by *R. planticola* DSMZ 4617 using various types of organic nitrogen source. (**A**), pullulanase activity; (**B**), starch concentration and (**C**), growth profile. Symbol: (◆), meat extract; (◇), tryptone; (□), tryptose; (●), yeast extract; (○), bacteriological peptone; (■), peptone from meat. Error bars indicate the mean ± standard deviation of triplicate.

Growth increased with increasing initial culture pH between 4 to 9 (Fig. **3B**). There was no significant difference for growth between pH 5 to 8 and growth reached a station-

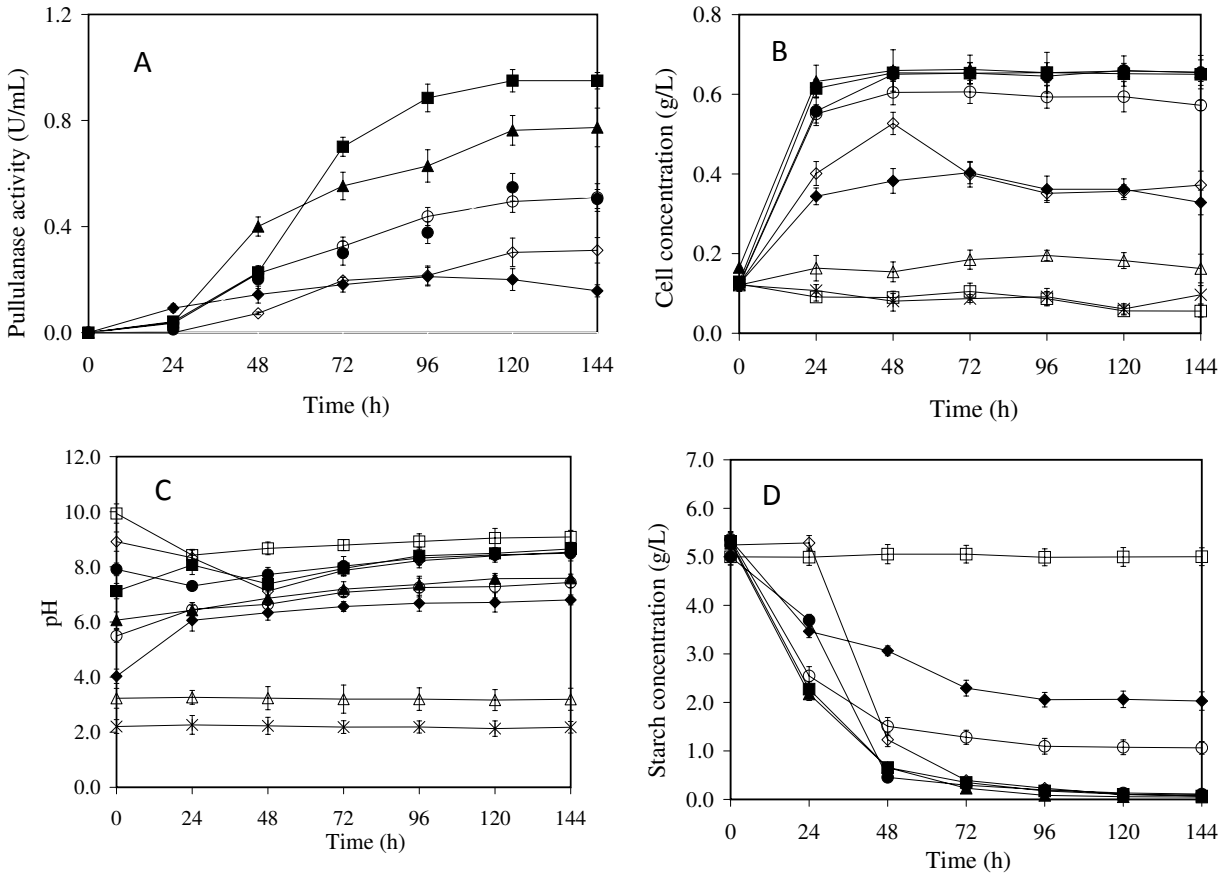

Fig. (3). Time course of pullulanase production by *R. planticola* DSMZ 4617 under different initial culture pH. (**A**), pullulanase activity; (**B**), growth profile; (**C**), pH and (**D**), starch concentration. Symbols: (*), pH 2; (△), pH 3; (◆), pH 4; (○), pH 5; (▲), pH 6; (■), pH 7; (●), pH 8; (◇), pH 9; (□), pH 10. Error bars indicate the mean ± standard deviation of triplicate.

Table 4. Comparison of the Performance and the Kinetic Parameter Value of Pullulanase Fermentation by *R. planticola* DSMZ 4617 in Shake Flask Using Different Types of Organic Nitrogen Source

Parameters	Organic Nitrogen Source					
	Meat Extract	**Tryptone**	**Tryptose**	**Yeast Extract**	**Bacteriological Peptone**	**Peptone**
P_{max} (U/mL)	$0.24^{b, c}$	0.87^{a}	0.36^{b}	0.40^{b}	0.18^{c}	0.95^{a}
t_p (h)	168^{a}	144^{b}	144^{b}	168^{a}	168^{a}	120^{b}
X_{max} (g /L)	0.65^{a}	0.67^{a}	0.67^{a}	0.70^{a}	0.55^{b}	0.64^{a}
t_x (h)	144^{a}	144^{a}	120^{b}	95^{c}	144^{a}	72^{b}
S (x 10^{-3} g/mL)	3.05^{d}	5.33^{b}	4.70^{c}	4.71^{c}	3.17^{d}	5.23^{a}
P (x 10^{-3} U/mL.h^{-})	1.43^{d}	6.25^{b}	2.5^{c}	2.38^{c}	1.09^{e}	7.92^{a}
$Y_{P/S}$ (U pullulanase/g starch)	78^{d}	169^{b}	77^{e}	85^{c}	58^{f}	182^{a}
P_{max}/X_{max} (U/g cell)	372^{e}	1343^{b}	539^{d}	576^{c}	335^{f}	1484^{a}

[a-f] Mean values in the same row not followed by the same letter are significantly different (P<0.05).

ary phase after 120 h of cultivation with maximum cell concentration of approximately 0.65 g/L. No growth or pullulanase production was detected in medium with initial culture pH less than 4 or more than 9, indicating that *R. planticola* DSMZ 4617 was neither an acidophilic nor alkalophilic pullulanase-producing bacterium.

The profiles of pH during pullulanase fermentation by *R. planticola* DSMZ 4617 at different initial culture pHs are shown in Fig. (**3C**). In fermentation with initial culture pH from 4 to 7, pH was increased during the early stages of cultivation. In contrast to this, for initial culture pH 8 and pH 9, pH was reduced to around 7.5 before the pullulanase was

Table 5. Comparison of the Performance and the Kinetic Parameter Value of Pullulanase Fermentation by *R. planticola* DSMZ 4617 in Shake Flask Using Different Types of Inorganic Nitrogen Source

Parameters	Inorganic Nitrogen Source		
	Sodium Nitrate	Ammonium Sulfate	Ammonium Acetate
P_{max} (U/mL)	0.95[a]	0.67[c]	0.18[c]
t_p (h)	120[b]	144[a]	120[b]
X_{max} (g/L)	0.64[a]	0.68[a]	0.45[b]
t_x (h)	72[b]	72[b]	72[b]
S (x 10^{-3} g/mL)	5.23[b]	5.46[a]	1.82[c]
P (x 10^{-3} U/mL·h)	7.92[a]	4.86[b]	1.46[c]
$Y_{P/S}$ (U pullulanase/g starch)	181[a]	128[b]	96[c]

[a-c] Mean values in the same row not followed by the same letter are significantly different (P<0.05).

Table 6. Effect of Different Initial Culture pH Values on the Performance of Pullulanase Fermentation by *R. planticola* DSMZ 4617 in Shake Flask

Initial pH	Parameters							
	P_{max} (U/mL)	t_p (h)	X_{max} (g/L)	t_x (h)	S (x 10^{-3} g/mL)	P (x 10^{-3} U/mL.h)	$Y_{P/S}$ (U/g starch)	P_{max}/X_{max} (U/g cell)
4	0.21[f]	96[c]	0.40[d]	72[a]	3.29[d]	2.20[e]	64.1[e]	523[f]
5	0.50[d]	120[b]	0.61[b]	72[a]	4.28[c]	4.20[d]	116.8[c]	825[d]
6	0.77[b]	144[a]	0.66[a]	72[a]	5.22[a]	5.38[b]	148.3[b]	1169[b]
7	0.95[a]	120[b]	0.66[a]	72[a]	5.23[a]	7.92[a]	181.6[a]	1435[a]
8	0.55[c]	120[b]	0.65[a]	72[a]	4.90[b]	4.57[c]	111.8[d]	843[c]
9	0.31[e]	144[a]	0.53[c]	48[b]	5.14[a]	2.08[e]	58.4[f]	588[e]

[a-f] Mean values in the same column not followed by the same letter are significantly different (P<0.05).

Notes:
P_{max} – Maximum extracellular pullulanase.
t_p – Time to reach maximum extracellular pullulanase.
X_{max} – Maximum cell concentration.
t_x – Time to reach maximum cell concentration.
S – Concentration of substrates utilized.
P – Pullulanase productivity.
$Y_{P/S}$ – Pullulanase yield.

detected in the culture fluid. In all cases, at the end of fermentation, the culture pH was in the range of pH 6 to pH 9. This indicates that the pH ranging from 6 to 9 was favorable for *R. planticola* DSMZ 4617 to secrete the enzyme outside the cells.

Starch hydrolysis rate increased with increasing initial culture pH and for medium with initial pH between 6 to 9, no significant difference for amount of starch hydrolyzed was observed (Table **6**). For medium with initial culture pH between 5 to 9, more than 80% of starch was hydrolyzed for abundant cell growth and pullulanase production. At pH below 4 and above 9, growth and starch hydrolysis was very poor. The observed yield of pullulanase ($Y_{P/S}$) was significantly different between each of the pH, as shown in the following sequence: pH 7 > pH 6 > pH 5 > pH 8 > pH 4 > pH 9. The value of P_{max}/X_{max} was also higher in medium with initial culture pH between 5 to 8 where more than 800 unit of enzymes produced per unit of cells.

CONCLUSION

In batch fermentation experiments using sago starch and peptone as carbon and nitrogen sources, we proved that pullulanase production by *R. planticola* DSMZ4617 could be enhanced. The pullulanase activities were 67% higher when sago starch was used to replace maltose in fermentation medium. In addition, initial culture pH and incubation temperature also had strong effects on the cell growth and pullulanase production. By applying initial culture pH of 7 and incubation temperature of 30°C in medium containing sago starch and peptone, significant high activity of pullulanase could be produced by *R. planticola* DSMZ 4617.

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