POTENTIAL USE OF AN EXTRACELLULAR ENZYME OF α-AMYLASE FROM INDIGENOUS INDONESIAN MESOPHILIC BACTERIA

Potensi Enzim α-Amilase dari Bakteri Mesofilik Indonesia

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ABSTRACT

Amylase enzyme has a great significance for industrial usages in Indonesia. However, this enzyme is still imported. The use of bacteria in biotechnological process of industrial products such as enzyme production has stimulated the exploration of extracellular amylase producing bacteria. This study aimed to identify and analyze the potential use of amylolytic bacterial enzymes for hydrolyzing cassava starch. Two bacterial isolates, i.e. MII-10 and DKW-8 originated from Indonesia soil were identified based on their morphological, physiological and biochemical properties according to the standard protocol. The isolates were then cultivated on fermentation medium and their growth pattern and enzymatic assays were observed. The acetone-precipitated crude enzyme harvested based on predetermined cultivation time was used for enzymatic hydrolysis product characterization on cassava starch using thin layer chromatography (TLC). The results showed that the mesophilic bacteria isolates (MII-10 and DKW-8) were belonged to *Bacillus licheniformis*. The maximum bacterial cell growth and enzyme activity were reached at 48 hours after incubation. The MII-10 isolate was found more stable than DKW-8 in producing amylase enzyme. Amylase produced by the MII-10 and DKW-8 isolates was identified to be an endo-α-amylase as confirmed by oligosaccharides and dextrin of the random hydrolysis products. Relatively high dextrose equivalence (DE) value of α-amylase of MII-10 (DE of 9.96) suggests that the enzyme is prospective for saccharification of starchy material in glucose syrup industry.

[***Keywords*:** α-amylase, mesophilic bacteria, starch, thin layer chromatography, Indonesia]

ABSTRAK

Enzim amilase mempunyai kegunaan penting dalam berbagai industri di Indonesia. Namun, enzim ini masih diimpor. Penggunaan bakteri dalam proses bioteknologi di bidang industri, seperti pada produksi enzim telah mendorong eksplorasi amilase dari bakteri mesofilik. Penelitian ini bertujuan untuk mengidentifikasi taksonomi bakteri amilolitik, parameter enzimatik, dan produk hidrolisis pati. Dua isolat bakteri (MII-10 dan DKW-8) yang berasal dari tanah di Indonesia diidentifikasi karakter morfologi, fisiologi, dan biokimianya berdasarkan protokol standar. Bakteri ditumbuhkan dalam fermentor, selanjutnya diamati pola pertumbuhan dan uji enzimatisnya. Enzim kasar dalam aseton yang dipanen berdasarkan waktu panen yang telah dioptimasi sebelumnya, dikarakterisasi produk hidrolisis enzimatisnya pada pati ubi kayu dengan kromatografi lapis tipis (KLT). Hasil penelitian menunjukkan bahwa bakteri mesofilik MII-10 dan DKW-8 termasuk dalam Bacillus licheniformis. *Pertumbuhan maksimum sel bakteri dan aktivitas enzim dicapai pada 48 jam setelah inkubasi. Bakteri MII-10 lebih stabil daripada DKW-8 dalam memproduksi enzim amilase, yang ditunjukkan oleh peningkatan aktivitas amilase sejak awal kultivasi. Amilase yang diproduksi oleh isolat MII-10 dan DKW-8 merupakan endo-α-amilase berdasarkan konfirmasi produk hidrolisis secara acak yang menghasilkan oligosakarida dan dekstrin. Nilai* dextrose equivalent *(DE) α-amilase dari MII-10 yang cukup tinggi (DE 9.96) menunjukkan bahwa enzim tersebut prospektif digunakan pada proses sakarifikasi bahan berpati dalam pembuatan sirup glukosa.*

[***Kata kunci:*** α-amilase, bakteri mesofilik, MII-10, pati, kromatografi lapis tipis, Indonesia]

INTRODUCTION

Amylases are important enzymes employed in starch processing industries for hydrolyzing polysaccharides such as starch into simple sugar constituents (Gupta *et al.* 2003). These starch degrading enzymes have received an attention because of the perceived technological significance and economic benefits. In recent year, the use of bacteria in biotechnological

process of industrial products such as enzyme production has stimulated the exploration of extracellular amylase producing bacteria (Van der Maarel *et al*. 2002; Pascon *et al*. 2011). Among amylolytic bacteria, *Bacillus* sp., in particular *B. subtilis*, *B. licheniformis*, and *B. amyloliquifaciens* are commonly utilized for producing amylases (Brena *et al*. 1996).

Amylases are grouped based on the chain of the attacked substrate by three types, i.e. α-amylase, β-amylase and amyloglucosidase. Amylases hydrolyze the starch molecules into polymers which are composed of glucose, maltose and a complete series of limit dextrins (Prakasham *et al*. 2007). Alpha-amylase breaks the polysaccharide chains quickly and randomly in the bond of 1.4-glycosidic, so-called endo-amylase. In contrast, β-amylase hydrolyzes glycosidic bonds in starch from the end (Gupta *et al*. 2003) and produces β-maltose and β-limit dextrins as main products. Whereas amyloglucosidase enzyme breaks non-reducing glucose to glucose. The enzyme can easily be distinguished from α-amylase and β-amylase (Nikolov and Reilly 1991).

The spectrum of amylase application has been widened in many other fields, such as clinical, medical and analytical chemistries, as well as in sugar syrup, textile, food, fermentation, and paper, brewing and distilling industries (Pandey *et al*. 2000). Alpha-amylase and amyloglucosidase are usually used for producing glucose through a natural bio-conversion (Omemu *et al*. 2005), which is called hydrolytic depolymerization of starch. Conversion of starch into fructose syrup as a sweetener by using enzymes from microorganisms is a relatively new industrial process and can increase the added value of starch.

Producing fructose syrup needs some steps by using α-amylase, amyloglucosidase and glucose isomerase (Whitehurst and Oot 2009). In liquid sugar industries, there are two important steps to produce glucose syrup, namely liquefaction and saccharification. The liquefaction process involves either α-amylase or β-amylase (Rao and Satyanarayana 2007), while saccharification process involves amyloglucosidase (Kobayashi *et al*. 1998). Alpha-amylase can also be added to the saccharification process and works synergistically with amyloglucosidase to speed up glucose formation (Satyanarayana *et al*. 2004). However, some critical factors are considered for industrial saccharification process, for instance, the poor stability of amylolytic activities and limited yields of glucose due to the slow hydrolysis of starch formation from condensation products. To encounter such cases, microbial enzymes that do not have these characteristics are highly desirable expected.

Application of α-amylase for enzymatic hydrolysis has now replaced acid hydrolysis which gains more magnitude among the industrial enzymes due to the importance of starch, sugar and other products in modern biotechnology era (Prakasham *et al*. 2007). Since the industrial starch degradation is usually initiated by α-amylase, several studies have been conducted using starchy materials. Various commercial raw starches (potato, wheat, corn, rice) are compared for their enzymatic hydrolysis products using α-amylase (Dincbas and Demirkan 2010) and the degrading ability between α- and β-amylases in raw starch granule (Sarikaya *et al*. 2000).

Indonesia has largely imported amylase for various industrial processes. On the other hand, cassava starch as a natural material for producing glucose syrup has not been intensively used. Considering the crucial need of α-amylase and highly starchy material production in Indonesia, study on α-amylase hydrolysis is needed.

Products of starch hydrolysis of α-amylase are used in various foodstuffs with different processes. Therefore, the hydrolysis test is valuable to be elucidated. Since α-amylase has been extensively used in starch bioconversion (Omemu *et al*. 2005), various studies on the enzymatic hydrolysis ability have been conducted (Rajagopalan and Krishnan 2008; Gangadharan *et al*. 2009). Several amylolytic *Bacillus* sp. are explored their hydrolysis abilities, such as *B. amyloliquefaciens* on soluble and commercial raw starches (Dincbas and Demirkan 2010), *B. brevis* (Ray *et al*. 2008), *B. subtilis* KCC103 (Rajagopalan and Krishnan 2008), as well as *B. licheniformis* (Shewale and Pandit 2007).

Information on amylase application with a view of their exploitation on enzymatic hydrolysis process using low cost-cassava starch in Indonesia is very essential. This study aimed to identify the potential use of amylolytic indigenous bacteria from Indonesian soil (MII-10 and DKW-8) and to investigate the cell growth pattern and enzymatic assays. Amylase produced by the bacteria was identified its ability on cassava starch and the product profile was determined by thin layer chromatography.

MATERIALS AND METHODS

Bacterial Isolates

Two amylolytic bacterial isolates (MII-10 and DKW-8) used in this study were obtained from the Indonesian Center for Agricultural Biotechnology and Genetic

Resources Research and Development (ICABIO-GRAD), Bogor, West Java. These bacteria were isolated from Indonesian soil. The isolates were maintained at 4°C in a starch nutrient agar slant medium containing 25 g l^{-1} of nutrient agar and 3 g l^{-1} of starch at pH 7.0.

Bacterial Identification

The isolates were cultured on starch-nutrient agar plates and incubated at 37°C for 24 hours. Single colonies of the isolates were repeatedly subcultured on fresh starch-nutrient agar plate to obtain pure cultures for identification and enzyme analyses. Bacterial identification followed the standard protocols such as Gram's staining, spore formation, and physiological and biochemical properties as described by Breed *et al*. (1994).

Amylolytic Enzyme Preparation

Single colonies of the isolates were cultured on an activation medium (50 ml) containing nutrient broth and 1% soluble starch and incubated at 37°C under shaking (120 rpm). After 24 hour incubation, the cultures were transferred into a 3 liter-fermented flask containing one liter fermentation medium broth equipped with an agitator and an aerator for 48 hours. The fermentation medium to produce amylase consisted of 0.1% yeast extract, 0.1% bacto trypton, 0.01% $MgCl_2$, 0.02% $CaCl_2$, 0.02% KH_2PO_4, 0.04% K_2HPO_4, 0.05% $(NH_4)_2SO_4$, 0.02% NaCl and 1% cassava starch. The optimum period for enzyme production was determined by sampling the culture at various times. Parameters analyzed were optical density (OD) of the cultures, enzyme activities, reducing sugar and soluble protein content (Aquino *et al*. 2003).

Enzyme Precipitation

The cultures were centrifuged at 4,000 rpm for 30 minutes at 4°C and the supernatants were collected. The cell-free supernatant was subsequently precipitated with cold acetone (4°C) at a ratio of 2:3. The mixtures were incubated for 12 hours at 4°C and centrifuged again to separate the sediment. The precipitated amylase was then dissolved with citrate phosphate buffer solution (pH 7.0) at a ratio of 1/50 times of supernatant volume.

Enzymatic Assays

Alpha-amylase and reducing sugar activities of the enzyme were determined using 3'5-dinitrosalicylic acid (Mamo and Gessesse 1997). One unit of amylase activity was defined as the amount of enzyme that produced 1 mmol α- or β-maltose per minute. Protein was estimated by Bradford method using bovine serum albumin as the standard (Kruger 1991).

Enzymatic Starch Hydrolysis

Starch liquefaction was carried out by preparing a substrate of cassava starch suspension (2%) in water, the pH was adjusted to 6.5 with $CaCO_3$, and heated until gelatinization. Two ml of the tested enzyme, approximately ranging from 400 to 500 U ml^{-1} (MII-10 and DKW-8), were added to 10 ml of the starch substrate and incubated at 40°C in a shaking incubator for a given period. Solution of hydrolysis product was separated and mixed with iodine solution. The mixture was boiled for 5 minutes and 2% of activated charcoal was added, then left for 1 hour and filtered. A commercial α-amylase from *B. licheniformis* was used as the standard (79 U/I α-amylase) followed the method described above.

Characterization of Hydrolysis Products

The starch hydrolysis products were analyzed by thin layer chromatography (TLC). The commercial pure of α-amylase was used as the standard for the hydrolysis test using cassava starch as the substrate. The test was also used for determination of dextrose equivalent (DE). In TLC assay, silica gel plates of 20 cm x 20 cm with a mobile phase system of isopropanol, acetone and lactic acid 0.1 M at a ratio of 4:4:2 were used. Sugar spots were detected by heating at 100°C for 30 minutes with color generating reagent consisted of a mixture of 4 ml aniline, 4 g α-diphenylamine, 200 ml acetone and 30 ml 80% H_3PO_4 (Schulz *et al*. 1986). Glucose, maltose and dextrin at 0.8% concentration each were used as standards.

The amounts of enzyme added to the substrate in the purified MII-10 and DKW-8 amylase, as well as in the pure α-amylase standard were prepared with the same unit (79 U) as enzyme per 0.2 g of starch. This value was the minimum ratio that produced brownish purple color with iodine reagent after 1 hour incubation. Starch dry weight was determined by heating the starch at 105°C for 4 hours and repeated once,

then drying using desiccator until reaching constant weight. Dextrose equivalence (DE) was determined by analyzing the reducing sugar as glucose according to the protocol of Takasaki *et al.* (1993).

RESULTS AND DISCUSSION

Characteristics of Amylolytic Bacterial Isolates

The macroscopical, microscopical, physiological and biochemical characteristics demonstrated that both isolates (MII-10 and DKW-8) were presumptively identified as *Bacillus licheniformis* (Table 1). The morphology of the isolates showed straight and rod shape as presented in Figure 1A. Both isolates were detected as mesophilic bacteria producing thermo-labile amylase and complement to previous isolated mesophilic amylolytic microbes (Ruban *et al.* 2013). Clear zones formed around the colonies on starch medium indicated that the isolates were able to produce extracellular starch-degrading amylase (Fig. 1B). The clear zones lead to select MII-10 and DKW-8 as isolates with the highest amylase activity.

To date, these novel amylolytic bacterial isolates in this study could enrich the discovery of *Bacillus* sp.

Table 1. Morphological, physiological and biochemical characteristics of MII-10 and DKW-8 isolates.

Parameters	Characteristics	
	MII-10	DKW-8
Cellular characteristics		
Morphology	Straight, rod shape	Straight, rod shape
Gram reaction	Gram positive	Gram positive
Endospore	Spore-forming	Spore-forming
Physiological characteristics		
Catalase	Positive	Positive
Indole production	Negative	Negative
Starch hydrolysis	Positive	Positive
Urease test	Negative	Negative
Oxidase test	Positive	Positive
Arabinosa hydrolysis	Negative	Negative
Mannitol hydrolysis	Negative	Negative
Xylose hydrolysis	Negative	Negative
Methyl red test	Negative	Negative
Motility	Positive	Positive
Growth:		
Anaerobic	Positive	Positive
At 50°C	Positive	Positive
At 60°C	Negative	Negative
At pH 5.7	Positive	Positive
In 7% NaCl	Positive	Positive

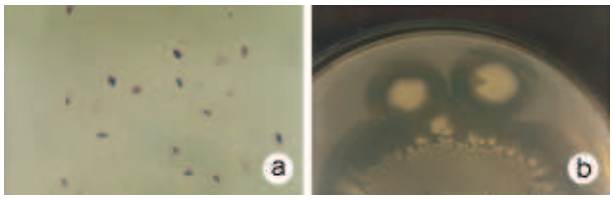

Fig. 1. Performance of amylase producing bacteria. (A) Bacterial cells showing straight, rod shape under light microscope observation, (B) amylase-producing bacteria showing a big clear zone around the colony on starch medium.

which have been confirmed elsewhere (Ajayi and Fagade 2006; Baishya and Sarma 2010). These results demonstrate that α-amylase producing bacteria are mostly from *Bacillus* sp. This is in a good agreement with the previous report (Niaz *et al.* 2010). The colony characters of both isolates could help their genetic characterization. Thus, the extracellular enzymes produced by *Bacillus* species have important roles at present. Of total estimated α-amylase production in the world, a half of this was produced by *Bacillus* sp. (Meima and van Dijl 2003). Among the *Bacillus* sp., α-amylase obtained from *B. licheniformis* is applied mainly for starch hydrolysis (Rao and Satyanarayana 2007).

Bacterial Growth and Amylase Production

The growth pattern, enzyme activity, reducing sugar and protein of MII-10 and DKW-8 isolates are presented at Figure 2. The MII-10 growth pattern determined by optical density showed that the lag phase lasted 2 hours and the exponential phase occurred approximately at 28 hours after incubation. Maximum cell growth and amylase activity reached at 48 hours of cultivation (621.9 U/I), but reducing sugar decreased slightly at 42-48 hours. While the DKW-8 growth pattern showed a lag phase of 2 hours and was accompanied by a sharp increase in enzyme activity. Afterward, the enzyme activity decreased until the end of incubation.

The MII-10 growth pattern indicates an increased amylase activity and protein since the beginning of cultivation, in contrast to reducing sugar. It is possibly due to the substrate used for cell metabolism. On the other hand, the protein of DKW-8 decreased continuously after reaching a peak at initial incubation. This assumes that MII-10 amylase is more stable than that of DKW-8. This result is consistent with previous studies (Ajayi and Fagade 2006)

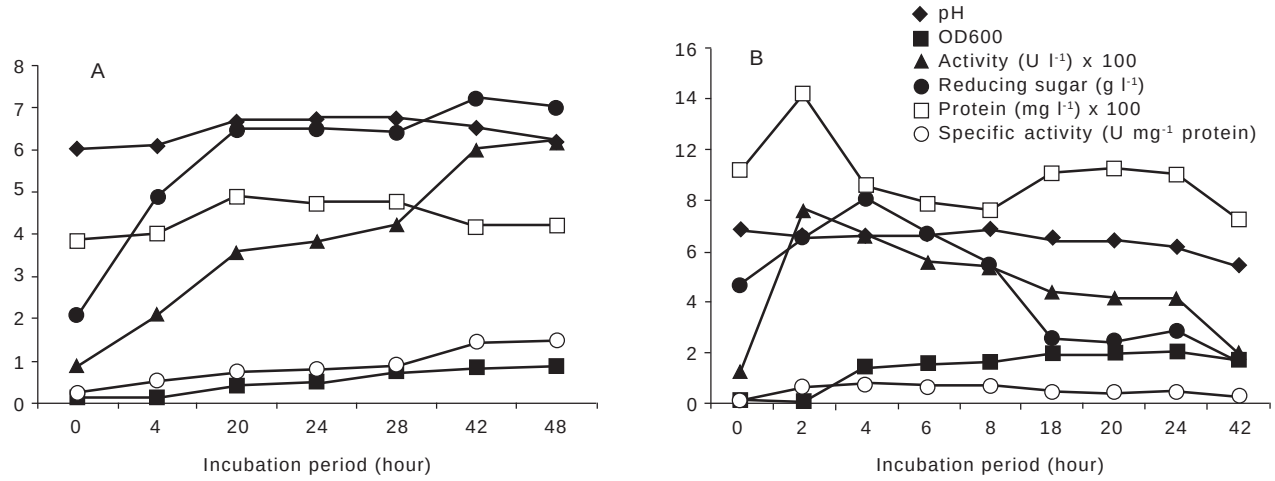

Fig. 2. Growth pattern, enzyme activity, protein, reducing sugar and specific activity of MII-10 (A) and DKW-8 (B) isolates during cultivation in fermenter broth medium.

confirming that the shape of the curve differs depending on the source of the enzyme.

The harvesting time of cell-free supernatant is determined by both enzyme activity and cell growth. The enzyme was harvested when the cell growth was stable and had high enzyme activity. In that phase, proteolytic enzyme production is still low, and neutral protease is peaked the day after amylolitic enzyme reaches the maximum value. Optimum growth of cells is linear with maximum growth rate, and amylase activity increases with cell growth (Riaz *et al.* 2009). The high stability of bacteria secreting amylase hopefully enhances a large amount of enzyme with higher activity.

Hydrolysis Product of Amylase

Hydrolysis product on cassava starch signified that amylase of MII-10 was better than that of DKW-8 at various incubation periods (Table 2). The purified amylase of MII-10 revealed a pretty good ability of hydrolysis on cassava starch. It could be seen in the initial 10 minutes of iodine test that gave a purple color, indicating that hydrolysis process had occurred. While amylase of DKW-8 took longer in hydrolyzing the starch, specified by purple color after 2 hours of hydrolysis.

Starch hydrolysis produces three types of dextrins, i.e. amylodextrin, erythrodextrin and acrodextrin. Amylodextrin produces a blue color when reacted with iodine and the longer hydrolysis would form erythrodextrin with brownish red color. In the shorter hydrolysis test, the color was usually formed with iodine and produced acrodextrin, which also called maltodextrin. The purple color in iodine staining can be caused by the mixed amylodextrin and erythrodextrin in the reaction. The prolonged hydrolysis would increase color intensity of spots, governing to obtain end-product of this amylase.

Table 2. Iodine test for hydrolysis product of amylases produced by MII-10 and DKW-8 isolates on cassava starch.

Incubation period (hour)	Color of hydrolysis products	
	MII-10	DKW-8
0	Blue	Blue
1/6	Purple	Blue
1/2	Purple	Blue
1	Purple	Blue-purple
2	Brown	Purple
24	Transparent	Transparent

Chromatogram patterns of enzymatic hydrolysis product by amylases of MII-10 and DKW-8 are presented in Figure 3 and 4, respectively. In the initial 10 minutes of hydrolysis, the main products of amylase from MII-10 were oligosaccharides 3 and dextrin. The intensity of color patches of oligosaccharide 2 increased within 30 minutes of hydrolysis. The DKW-8 chromatogram also showed an increased intensity of color patches by increasing hydrolysis duration. The extended hydrolysis (24 hours) resulted in an accumulation of maltose, dextrin and oligosaccharide 1 and reduced color intensity of oligosaccharides 2 and 3. Final products of MII-10 α-amylase varied, similarly to the standard α-amylase which produced glucose trace, maltose, oligosaccharides

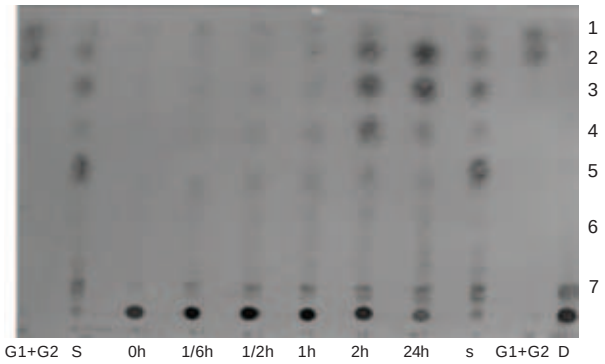

Fig. 3. A thin-layer chromatography plate showing main products of soluble starch hydrolyzed by amylase produced from MII-10 bacterial isolate. Lane 1 and 10 = G1+G2, mixture of glucose and maltose, lane 2 and 9 = S, standard α-amylase *Bacillus licheniformis*, lane 3-8 = the products catalyzed by MII-10 at 0,1/6, 1/2, 1, 2, and 24 hours, lane 11 = dextrin, No. 1-7 represent the hydrolysis products, 1 = glucose, 2 = maltose, 3-6 = malto-oligosaccharides, 7 = dextrin.

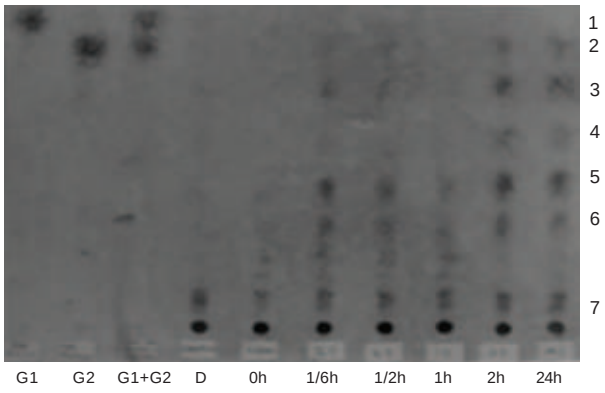

Fig. 4. A thin-layer chromatography plate showing main products of soluble starch hydrolyzed by amylase produced from DKW-8 bacterial isolate. Lane 1 = G1, glucose, lane 2 = G2, maltose, lane 3 = G1+G2, mixture of glucose and maltose, lane 4 = D, dextrin, lane 5-10 = the products catalyzed by DKW-8 isolate at 0, 1/6, 1/2, 1, 2, and 24 hours. No 1-7 represent the hydrolysis products, 1 = glucose, 2 = maltose, 3-6 = malto-oligosaccharides, 7 = dextrin.

and dextrin. This random hydrolysis product indicates an endo-α-amylase action. The α-amylase of DKW-8 is also an endo-α-amylase, however, hydrolysis product of amylase DKW-8 was not glucose for total period of hydrolysis. This result demonstrates that the hydrolysis rate of MII-10 amylase is higher than that of DKW-8. The discrepancy of hydrolysis products of amylase produced among bacteria is in accordance with previous studies. Endo-α-amylases from *B. licheniformis* were maltose, maltotriose and maltopentaose (Ratanakhanokchai *et al.* 1992) as well as maltohexaose (Santamaria *et al.* 1999) and also glucose (Aquino *et al*. 2003; Dincbas and Demirkan 2010).

DE of MII-10 α-Amylase

The stability of bacterial cell growth and the production of MII-10 amylase lead to determine its DE that represents the degree of hydrolysis on starch. The DE of MII-10 α-amylase precipitated with acetone was 9.96, lower than that of pure α-amylase standard from *B. licheniformis* (23.33). It could be because this imported standard α-amylase is a commercial product which was genetically modified bacteria with extreme increased activity. The DE of MII-10 amylase was comparable with that of other α-amylase (Sun *et al*. 2010). In fact, saccharification process in the manufacture of glucose syrup usually uses starch substrates with DE ranging from 10 to 15 (Nikolov and Reilly 1991). Based on the DE value, therefore, MII-10 amylase is potential for starch hydrolysis to produce glucose.

Utilization of Thermolabile MII-10 α-Amylase

Many factors affect the success of liquefaction and saccharification processes in industrial glucose syrup production, for instance substrate concentration, pH, temperature and stirring (Takasaki *et al*. 1993). This thermolabile MII-10 α-amylase could be used to break down cassava starch and economically produced glucose. The process of hydrolyzing starch and its derivatives which did not require high temperatures, particularly saccharification for producing liquid sugar, could be possibly applied. Some thermolabile amylases were also proven to apply in starch saccharification (Singh *et al*. 2010). The characters of thermolabile amylase in this study may provide an efficient way to produce glucose syrup from starch, by conducting further studies on amylolytic complex of *B. licheniformis.*

Further studies to optimize fermentation conditions for both isolates and to modify the genetics would enhance α-amylase activity to be a potential enzyme source. Importantly, as a large amount of foreign exchange is involved to import such enzymes to meet domestic consumption, serious attention and efforts are required to produce the indigenous amylase by using locally improved isolates.

CONCLUSION

Based on the morphological, physiological and biochemical characteritics, the two mesophilic bacteria isolates MII-10 and DKW-8 were identified as belonging to *Bacillus licheniformis.* The bacterial cell growth and amylase enzyme activity of the two isolates were found to be positive linear correlation. The highest amylase activity produced from both isolates was noticed at 48 hours after cultivation and determined as the optimum time to harvest the bacterial cells. MII-10 isolate was more stable compared with DKW-8 to produce amylase. The enzyme of MII-10 and DKW-8 isolates was identified as endo-α-amylase. The DE value indicates that MII-10 isolate should be more prospective than DKW-8 to produce thermolabile α-amylase. Thus, MII-10 α-amylase could be potentially applied in starch saccharification process to break starch into simple sugar in glucose syrup manufacture.

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