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Screening and characterization of amylase enzyme in sweet orange (*Citrus sinensis*) juice clarification

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Abstract. *Utami R, Widowati E, Christy A. 2016. Screening and characterization of amylase enzyme in sweet orange (*Citrus sinensis*) juice clarification. Nusantara Bioscience 8: 268-272.* The amylum compound of sweet orange cloudify the produced juice. Clarification by amylase could remove the cloudiness. The aimed of this study were screening the amylolytic bacteria from spoiled potato which are potential in clarification of sweet orange juice, and characterization of the resulted amylase (optimum pH and temperature, pH and thermal stability, KM and Vmax). The results showed that from thirteen bacterial isolates, three isolates (K-7, K-8, and K-11) produced enzymes that showed the best clarification activity on the sweet orange juice. The optimum pH of the isolate K-7, K-8, and K-11 enzymes were found to be 7, 7 and 6, respectively. The optimum temperature of the isolate K-7, K-8, and K-11 enzymes were found to be 35, 40 and 35°C, respectively. Enzymes of isolate K-7, K-8, and K-11 were stable in the pH range 3-6, 5-6, and 7-8, respectively. The enzyme of isolate K-7 and K-11 were stable at temperature 40-60°C while enzyme of isolate K-8 stable at temperature 40-70° C. Km values of isolate K-7, K-8, and K-11 enzymes were 0.2756 mg/mL, 0.0888 mg/mL, and 0.0956 mg/mL, respectively. Vmax values of isolate K-7, K-8, and K-11 enzyme were 0.0325 U/mL, 0.0164 U/mL, and 0.0134 U/mL.

Keywords: amylase; clarification; enzyme; juice; sweet orange
Abbreviation: TSS = total soluble solids, DNS = dinitro salicylic acid

INTRODUCTION

Sweet orange (*Citrus sinensis*) widely planted in Pacitan, East Java (Indonesia) so that it is known as Pacitan orange. According to data from Badan Litbang Pertanian of Indonesia, a total number of sweet orange production in Indonesia are 5-60 kg/trees/year. Moreover, data from Susenas in 2002, showed that total consumption of sweet orange is 1,2 kg/capita/year, so it takes a production of 240.000 tons per year of fresh fruit or 20.000 tons per month of processed fruit. Sweet orange has the sweetest taste among the group (Sutopo 2011). This makes a lot of sweet oranges used for the manufacture of fruit juice because it tastes sweet and fresh.

The definition of fruit juice beverage products (fruit juice) according to SNI 01-3719-1995 is a soft drink made from fruit juice and water with or without the addition of sugar and permitted food additives. While based on the Decree of the Head National Agency of Drug and Food Control No. HK. No. HK.00.05.52.4040 of 2006 on Food Categories governing the definition and basic characteristics of fruit juice, related to the provision of raw materials, processing, and finished products, fruit juice is a liquid obtained from the edible part of the fruit that was washed, crushed, clarified (if needed), with or without pasteurization and packaged to be consumed immediately.

One of the factors affecting the turbidity on fruit juices is starch. Immature apples contain 15% of starch. Although at first centrifugation process (before the juice into the clarification tank) can eliminate almost all of the starch content, but usually there was still 5% starch left. Agricultural Research Service of the US Department of Agriculture (1956) reported that the orange juice contains 76% carbohydrates of total dissolved solids. The content of starch in orange juice lies in the juice sacs.

Amylase is an enzyme that breaks down starch. These enzymes degrade starch and polymer related to the product yield of amylolytic enzymes. Amylase refers to an enzyme that capable of hydrolyzing the bond α-1-4-glycosidic of amylose, amylopectin, glycogen and degradation products (Aiyer 2005). According to Bhat (2000), α-amylase and amyloglucosidase used on fruits that contain high starch like apples to prevent the formation of fog in the beginning process of clarifying whereas cellulase enzymes were used in the mechanical process (pressing, centrifugation, and filtration).

This study will apply the amylase enzyme produced by amylolytic bacterial isolates from spoiled potato in sweet orange juice clarification. The purpose of this study was screening the amylolytic bacteria from spoiled potato which are potential in clarification of sweet orange juice, and characterization of the resulted amylase (optimum pH and temperature, pH and thermal stability, KM and Vmax).

MATERIALS AND METHODS

Materials

Spoiled potato waste was taken from Pasar Gede traditional market, Surakarta (Central Java, Indonesia). Clarification of orange juice using sweet citrus fruits obtained from Pasar Gede, Surakarta. Sweet oranges which

used are a ripe orange with orange peel greenish yellow color, the texture of the skin is not too thick, and citrus fruit size was standardized.

Screening of amylase producing bacteria for orange juice clarification

Each amylolytic bacterial isolate (1) mL was inoculated into 10 mL of liquid starch soluble medium and incubated for 24 hours. Crude enzymes were collected by centrifugation the medium at 6000 rpm for 15 min at 4°C. Crude enzyme activity was analyzed by the DNS method (Gusakov et al. 2011).

Sweet citrus fruits were washed thoroughly with running water. Extraction of sweet orange was using a lemon squeezer manually. Orange juice is then filtered using a filter cloth to separate the pulp. Soluble starch 1% was prepared to determine the activity of starch depolymerization by crude amylase enzyme. Orange juice and 1% soluble starch liquid (30 mL) were added with the crude enzyme of each test isolate (3 mL). Incubation was performed at 50°C for 60 minutes. The viscosity values (cp), the transmittance (% T) and TSS (°Brix) of orange juice were determined while 1% soluble starch was being specified its liquid viscosity (cp) and transmittance (% T).

Partially purified amylase production

Stocks of inoculum of selected isolates (10%) were inoculated on enzyme production medium. Agitation with a speed of 144 rpm was carried out until logarithmic phase was in accordance with the isolates growth curve (Widowati et al. 2014).

Isolates culture medium in logarithmic phase were centrifuged at 4°C with a speed of 6000 rpm for 15 minutes. The supernatant was precipitated with 50%-90% ammonium sulfate fractionation to separate enzyme. Furthermore, the solution was centrifuged at a speed of 8000 rpm at 4°C for 10 minutes. Enzyme activity test performed on the supernatant phase with the DNS method. Saturation fractions with the highest enzyme used in the further test. Dialysis performed by soaking the cellophane membrane bag in 300 mL of 0.02 M of citrate phosphate buffer pH 7 in 600 mL beaker glass and stirred with a magnetic stirrer for 24 hours in a cooling chamber at a temperature of 4°C. The enzyme activity of partially purified amylase was analyzed using the DNS method (Gusakov et al. 2011).

Characterization of partially purified amylase

Optimum pH

Partially purified amylase (0.1 mL) was added with 0.9 mL of reagent (0.7% (w / v) soluble starch and citrate phosphate buffer solution 0.02 M) at pH 5; 6; 7; 8 and 9. The solution was incubated at 37°C for 30 minutes. Samples were analyzed by the DNS method.

Optimum temperature

Partially purified amylase (0.1 mL) was added with 0.9 mL of reagent (0.7% (w / v) soluble starch and a solution of 0.02 M citrate-phosphate buffer pH 7) at an incubation temperature 35; 40; 45; 50; and 55°C for 30 minutes. Samples were analyzed by the DNS method.

Enzyme stability

pH stability of the partially purified amylase performed with the enzyme was incubated in 0.1 mL of 0.1 M phosphate buffer pH at 3,4,5,6,7,8,9 and 10 for 30 min at 50°C. Temperature stability of the enzyme performed with 0.1 mL enzyme was incubated in 0.1 mL of 0.02 M citrate-phosphate buffer pH 7 for 30 minutes at a temperature of 30,40,50,60,70,80,90, and 100°C. The enzyme activity was analyzed by the DNS method.

Analysis of enzyme kinetics

Partially purified amylase (0.1 mL) was added with 0.9 mL of reagent (0.7% (w / v) soluble starch [S] according to treatment is 0.05; 0.1; 0.2; 0.4 and 0, 8 mg / mL and a solution of 0.02 M citrate phosphate buffer pH 7). The solution was incubated at 37 ° C for 30 minutes. Samples were analyzed by the DNS method.

Km and Vmax values were determined by curve 1/ [S] versus 1/activity of the enzyme in order to obtain a linear line with a $1/v = 1/Vmax + Km/Vmax$. 1/ [S] or $y = a + bx$ with Y = 1/ activity of the enzyme or 1/enzyme speed (1/v); X = 1/substrate concentration (1/[S]). So as to determine Vmax and Km (1/2 Vmax) is $a = 1 / Vmax$ then $Vmax = 1 / a$; $b = Km.Vmax$ then $Km = b.Vmaks$ Windows.

RESULTS AND DISCUSSIONS

Screening of amylase producing bacteria for orange juice clarification

The highest enzyme activity possessed by isolates of K-11 that was equal to 0.0746 U/mL while the lowest enzyme activity possessed by isolates of K-20 (0.0150 U/mL) (Table 1). Isolates K-11 showed the higher final cell number (5.74 x 105 cells/mL) while isolates of K-8 showed the lower final cell count (9.00 x 104 cells/mL). Haribhau et al. (2015) isolated amylase producing microorganisms from soil and found that the enzyme activity ranged from 0.100 up to 0.501 U/mL.

The viscosity of amylase-treated orange juice samples ranged from 1.1113 to 1.1767 cp which lower than control sample (1.1680 cp). Transmittance (%T) of amylase-treated orange juice samples ranged from 0.7 to 0.8%, while control sample showed 0.8%. TSS (°Brix) of amylase-treated orange juice samples were 7 °Brix while control sample showed 8°Brix (Table 1). This indicated that amylase addition on orange juice performed clarification ability. The juice clarification by amylase related to starch content reduction. Carrin et al. (2004) also reported that amylase reduced the starch content in apple juice.

Isolates K-7 and K-8 were screened for future treatments because of high enzyme activity and its ability in sweet orange juice clarification. K-11 isolates were selected for future treatments based on the number of cells that eventually the highest and relatively rapid growth compared to other isolates in addition to the high enzyme activity and ability in sweet orange juice clarification.

Table 1. Enzyme activity of crude enzyme, cell number and clarification ability of crude enzymes

Isolate	Enzyme activity (Unit/mL)	Final cell number (Cell/mL)	Orange juice			Solubles starch liquid (1%)	
			Viscosity (cp)	%T	TSS (°Brix)	Viscosity (cp)	%T
K-1	0.0205	3.18×10^5	1.1147	0.8	7	4.8915	95.7
K-3	0.0211	2.50×10^5	1.1185	0.7	7	4.2319	96.7
K-4	0.0162	1.00×10^5	1.1204	0.8	7	5.2312	94.5
K-5	0.0222	2.14×10^5	1.1567	0.7	7	5.0898	96.1
K-7	0.0272	1.82×10^5	1.1113	0.7	7	4.2800	97.2
K-8	0.0270	9.00×10^4	1.1268	0.8	7	6.1745	96.8
K-10	0.0220	3.22×10^5	1.1767	0.8	7	5.5389	95.2
K-11	0.0746	5.74×10^5	1.1195	0.8	7	5.0219	96.3
K-12	0.0159	2.54×10^5	1.1379	0.8	7	5.6215	94.7
K-13	0.0184	1.50×10^5	1.1342	0.7	7	4.7676	95.8
K-14	0.0164	9.20×10^4	1.1268	0.8	7	4.6499	94.9
K-20	0.0150	1.76×10^5	1.1308	0.8	7	5.0898	95.9
K-21	0.0198	3.54×10^5	1.1149	0.8	7	6.1745	95

Table 2. Enzyme activity in various stages of purification

Isolate	Enzyme Activity (U/mL)		
	Crude	Ammonium sulphate precipitation	Dialysis
K-7	0.0272	0.0719	0.2904
K-8	0.0270	0.0679	0.1305
K-11	0.0746	0.0863	0.1880

Partially purified amylase characterization

Enzymes of K-7, K-8, and K-11 isolates were selected and partially purified by ammonium sulfate precipitation and dialysis methods. The enzymes activity at each purification step showed at Table 2. Enzyme activity after dialysis procedure showed the highest value. Purification fold of the ammonium sulfate precipitation followed by dialysis of crude enzymes ranged from 2.52-10.67. Singh et al. (2014) reported that after ammonium sulfate precipitation and dialysis, amylase recovered 56.58% with 2.98-fold purification. Mageswari et al. (2012) stated that purification fold of amylase from *Pontibacillus chungwhensis* strain P1 were 10.1 after ammonium sulfate precipitation and dialysis.

Enzymes of K-7 and K-8 isolates have the same optimum pH of 7.0, while the enzyme of K-11 isolates has a pH optimum of 6.0 (Figure 1). Several studies also reported that amylase from marine bacterium *Zunongwangia profunda* (Wu et al. 2014), *Bacillus methylotrophicus* strain P11-2 (Xie et al. 2014), and *Bacillus laterosporus* (Kumar et al. 2013) showed optimum activity at pH 7.0. Dutta et al. (2006) mention that the activity of amylase showed at pH 3.5 - 8.5 with maximum activity at pH 6.0. Enzymes of K-7 and K-11 isolates have the same optimum temperature of 35°C while the isolate of K-8 has an optimum temperature at 40°C (Figure 2). Samanta et al. (2013) stated that the amylase showed maximum activity at 37°C at pH-8.

The enzyme of the K-7 isolate was stable at pH 3-6 and inactive at pH 11. The enzyme of K-8 isolates was stable at pH 5-6 and inactive at pH 11. The enzyme of K-11 isolates was stable at pH 7-8 and inactive at pH 11 (Figure 3). The pH changes not only affected the form of the enzyme but also might change the shape or charge properties of the substrate for that reason the substrate cannot bind to the active side and can't catalyze. The enzyme of K-7 isolates was stable at temperatures of 40-60°C, and the activity begins to decline at 70°C and inactivated at a temperature of 100°C. The enzyme of K-8 isolates was stable at a temperature of 40-70°C, and the activities begin decreased at a temperature of 80°C and inactivated at 100°C. The enzyme of the K-11 isolate was stable at temperatures of 40-60°C, and the activity begins to decline at a temperature of 70°C and inactivated at a temperature of 100°C (Figure 4). The stability of the enzyme at pH and temperature parameters need to know due to the circumstances when the enzyme was inactive or not show activity anymore, and it is also to determine whether the enzyme can be combined with other enzymes. Mohamed et al. (2009) reported that the thermal stability of the α-amylase enzyme was stable up to 50°C, but some others were stable at 40°C. Samanta et al. (2013) stated that the amylase was stable within the pH 6 - 8 and temperature 30°C - 40°C.

Analysis of enzyme kinetics

Analysis of the kinetics enzymatic reactions include maximum reaction rate (Vmax) and Michaelis-Menten constant (Km). Michaelis and Menten concluded that the rate of reaction depends on the substrate concentration [S], then the addition of substrate concentration will result in the rate of reaction will be described when a straight line (Poedjiadi 2009). Km values showed affinity to the substrate and enzyme specific for each enzyme. The smaller the value of Km, the greater the affinity.

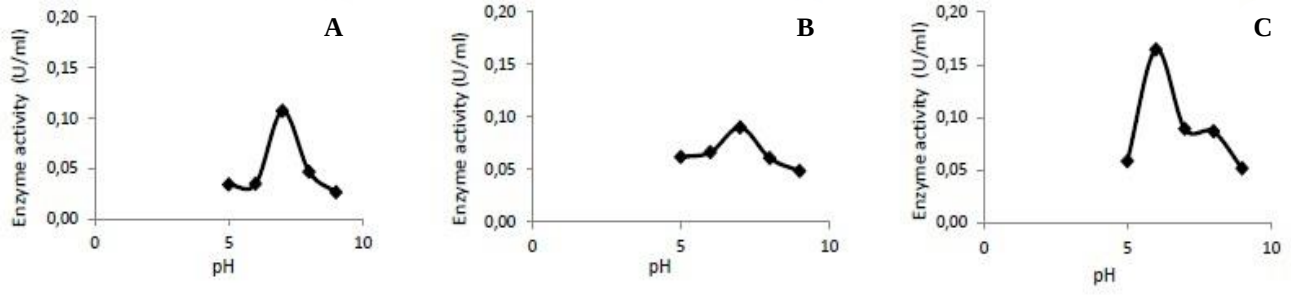

Figure 1. pH optimum of K-7 (a), K-8 (b) and K-11 (c) isolates enzyme

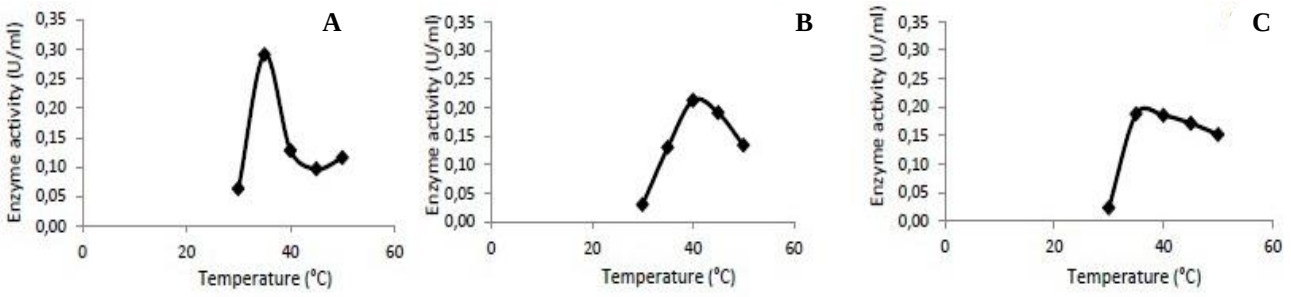

Figure 2. Temperature optimum of K-7 (a), K-8 (b) and K-11 (c) isolates enzyme

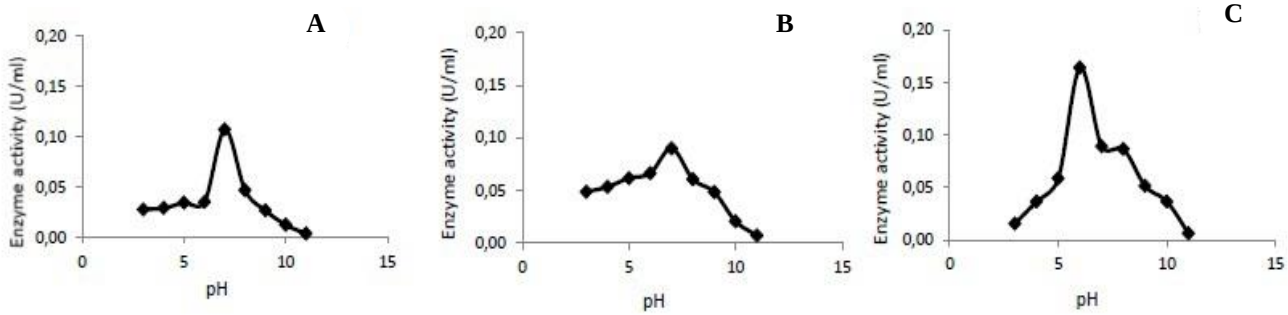

Figure 3. pH stability of K-7 (a), K-8 (b) and K-11 (c) isolates enzyme

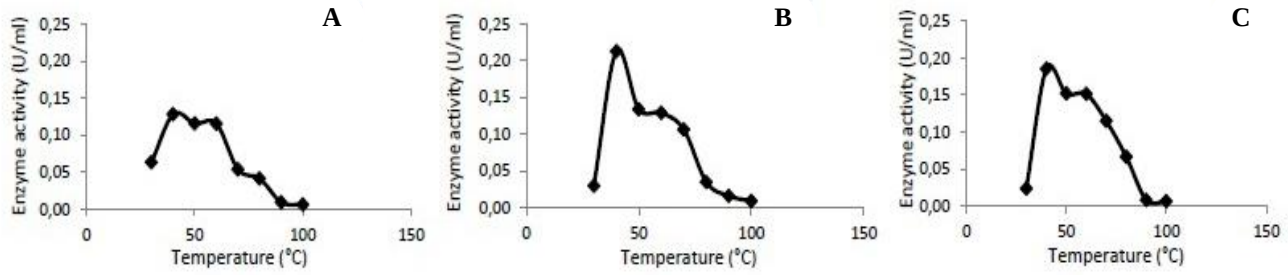

Figure 4. Temperature stability of K-7 (a), K-8 (b) and K-11 (c) isolates enzyme

Vmax values of each isolate demonstrated that the maximum product generated by the enzyme extract every minute of it. The enzyme of K-7 isolates showed maximum yield 0.1754 U/mL per minute. The enzyme of K-8 isolates showed maximum yield 0.0981 U/mL per minute. The enzyme of K-11 isolates showed maximum yield of 0.0725 U/mL per minute. The data are shown in Table 3, the enzyme of K-7 isolates showed the highest Km and Vmax values whereas isolates of K-8 enzyme showed the smallest Km value. Therefore, an enzyme of K-7 isolates has a low affinity for starch substrate while the enzyme of K-8 isolates has a high affinity for starch substrate. Amutha and Priya (2011) reported that Km values and Vmax values of amylase from *Bacillus subtilis* KCX 006 were 0.291 mg/mL and 23.69 U/mL, respectively. Enzymes with greater affinity to the substrate having a more significant role in the clarification of sweet orange juice. So, it can be concluded that the enzyme of K-8 isolates is recommended for industrial clarification of juice because it showed the lowest Km.

In conclusion, the results revealed that from thirteen bacterial isolates, three isolates (K-7, K-8, and K-11) produced enzymes that showed the best clarification activity on the sweet orange juice. The optimum pH of the isolate K-7, K-8, and K-11 enzymes were found to be 7, 7 and 6, respectively. The optimum temperature of the isolate K-7, K-8, and K-11 enzymes were found to be 35, 40 and 35°C, respectively. Enzymes of isolate K-7, K-8, and K-11 were stable in the pH range 3-6, 5-6, and 7-8, respectively. The enzyme of isolate K-7 and K-11 were stable at temperature 40-60°C while enzyme of isolate K-8 stable at temperature 40-70° C. Km values of isolate K-7, K-8, and K-11 enzyme were 0.2756 mg/mL, 0.0888 mg/mL, and 0.0956 mg/mL, respectively. Vmax values of isolate K-7, K-8, and K-11 enzyme were 0.0325 U/mL, 0.0164 U/mL, and 0.0134 U/mL.

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