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The Characterization of Amylolytic Enzyme Present in Fermented Sweet Sap of Palmyrah

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ABSTRACT

Amylase enzyme obtained from 12h of naturally fermented sweet sap of palmyrah. The amylase showed zero order kinetics for 10min. The optimum temperature for the activities of enzyme as 65^{o}C and pH was 7.0. When the enzyme was pre-incubated at 65^{o}C and at pH 7.0, it lost 62% of its initial activity at 60min. This enzyme showed activity with starch, lactose, maltose, pectin and sucrose. Amylase activity was strongly inhibited by 2mM of Hg^{2+} but 2 mM of Ca^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Na^{+}, Zn^{2+}, Mn^{2+} and Cu^{2+} stimulated the enzyme activity at 65 ^{o}C and at pH 7.0. The enzyme in the presence of 2mM Mn^{2+}, Cu^{2+} and Na^{+} separately, retained 79, 86 and 68 % of its initial activity respectively at 60 min, 65^{o}C and pH 7.0.

1. INTRODUCTION

Amylolytic enzymes form a large group of enzymes operating on starch and related oligo- and polysaccharides. The three best known amylases are α-amylase, β-amylase and glucoamylase (rarely γ-amylase). The amylases of microorganisms have a broad spectrum of industrial applications as they are more stable than when prepared with plant and animal amylases [1]. The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity and the fact that they are easily manipulated to obtain enzymes of desired characteristics [2]. Amylases are derived from several fungi, yeasts and bacteria [3, 4, 5 and 6]. Unfermented sweet sap of palmyrah (*Borassus flabellifer*) palm commonly referred to as 'sweet toddy' contains 10-16.5 % w/v sugar, mainly in the form of sucrose [7], vitamins and minerals forms a good culture medium for the microorganisms to grow. The crude sugar (jaggery) in Sri Lanka is mainly obtained from the sweet sugary sap obtained from the tapped inflorescence of the Coconut (*Cocos nucifera*), Palmyrah (*Borassus flabellifer*) and Kithul (*Caryota urens*) palms [8] The microorganisms usually seen in Palmyrah toddy are yeast (mostly *Saccharomyces cerevisiae*, *S. chevalieri*, *Kloeckere apiculata*, *Schizosaccharomyces pombe*) and bacteria (*Bacillus cereus*, *B. sphericus*, *B. firmus*). Fermented sweet sap of palmyrah (Toddy) is the natural fermented sap and contains about 5-6% (w/v) ethanol during taping of the sap. The objectives of this study were to extract amylolytic enzyme from naturally fermented sweet sap of palmyrah palm and characterization of the amylolytic enzyme.

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2. MATERIALS AND METHODS

2.1 Source of amylolytic enzyme

Palmyrah palms growing widely in Jaffna district of Sri Lanka was selected. Matured palm trees were chosen randomly in six different areas of the Jaffna district. Tips of young inflorescence of the palm trees were tapped and sterile, clean collecting pots were fixed. After 18 hours of collection of palmyrah sweet sap the samples were transferred to sterile bottles and brought to laboratory of department of botany, university of Jaffna. 100mL of sugary sap were transferred to 500mL of Erlenmeyer flasks and allowed to natural ferment for 12h at room temperature (29 ± 3) with shaking at 100 rpm. The filtrates were used as source of amylolytic enzyme sample.

2.2 Measurement of amylase activity

Soluble starch ($2gL^{-1}$) in 0.0lM phosphate buffer (pH 7.0) was pre incubated for 3 min at 65°C. Then 0.5mL of the enzyme sample was mixed with 0.5mL substrate and incubated for 5min at 65°C. Reducing sugar was measured by the DNS method [9]. One unit of amylase activity is defined as the amount of enzyme that produces one Mole of reducing sugar in one minute at 65°C, and pH 7.0 from soluble starch ($20gL^{-1}$) as substrate.

2.3 Effect of time

Soluble starch (0.5mL, $20gL^{-1}$ in phosphate buffer pH 7.0) was allowed to react with amylase enzyme sample (0.5mL) at 65°C and the amount of glucose produced was monitored. The time suitable for the incubation was optimized.

2.4 Effect of temperature

The effect of temperature on enzyme sample was determined by incubating enzyme for optimized time with 0.5mL of soluble starch ($20gL^{-1}$) at different temperatures, varied from 30 to 75°C. Then activities of the enzyme samples were measured and relative activities were calculated.

2.5 Effect of pH

The effect of pH on activity of enzyme sample was measured by preparing $20gL^{-1}$ soluble starch in buffers of different pH values ranging from 4.0 to 10.0 (for pH from 4.0 to 6.0 citrate phosphate buffer, for pH 8.0 Tris buffer, for pH 9.0 glycine NaOH buffer and for pH 10.0 carbonate, bicarbonate buffer were used). Enzymes were incubated at optimized temperatures for optimized period at 65°C.

2.6 Stability of enzymes with temperature

Amylolytic enzyme was pre-incubated at 65°C and at pH 7.0 and the activities of the enzymes were monitored.

2.7 Substrate specificity of the enzyme

Starch, Lactose, Maltose, Pectin and Sucrose of $20gL^{-1}$ concentration in 0.01M phosphate buffer (pH 7.0) were prepared and were used as substrates. The activities of the amylolytic enzymes were determined at 65°C.

2.8 Effect of cations on the activity and stability of the enzyme

To determine the effect of 2 mM cations such as Zn^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Na^{+}, Hg^{2+}, Ca^{2+} and Cu^{2+} on the activity of amylase from fermented sweet sap of palmyrah 2mM ions (Ca^{2+}, Mg^{2+}, Na^{+}, Hg^{2+}, Mn^{2+}, Zn^{2+} and Ba^{2+} in the form of chlorides and 2 mM Cu^{2+} in the form of sulphate) enzyme solutions were prepared separately and the enzyme activities were measured.

To determine the thermal stability of enzyme containing 2mM Mn^{2+}, Cu^{2+} and Na^{+} were pre incubated separately at 65°C for 1 hour and the enzyme activity were monitored.

3. RESULTS AND DISCUSSION

The phloem sap of Palmyrah palm (*Borassus flabellifer*) contains many nutrients such as nitrogen, protein, sugars, minerals as ash phosphorus, iron, vitamin C, vitamin BI [10] therefore it is an ideal medium for the growth of microorgansms. Its neutral pH favours the growth and multiplication of bacteria. Initially the fermenting sweet sap had a bacteria count around 124 cells/mL, while the yeast cell count was 56 cells/mL. After natural fermentation at 100rpm and roomtemperature (29 ± 3) for 12 hours, the yeast cell count increased up to 136 cells/mL while the bacteria count decreased to 73 cells/mL. Similar microbial changes had been observed in the cocount sap [11], Kithul palm [12] and palm wines [13] during natural fermentation.

Today a large number of microbial amylases are available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industry. Several yeast strains produce amylolytic enzyme under different fermentation conditions [14, 15 and 16]. In our study presence of amylolytic enzyme in fermented sweet sap of palmyrah was confirmed by measuring the activity of amylase enzyme. The amylase enzyme production could be due to activity of yeast cells.

3.1 Effect of time

The influence of incubation time on the production of glucose from the reaction of amylase with starch ($20gL^{-1}$) was studied for 1h at pH 7.0 and 65°C. Amylase preparations showed a linear relationship between the time and production up to 10minutes. Hence, it was decided to fix the reaction time for 5min.

3.2 Effect of temperature on the activity of amylase enzyme

The initial relative activity of amylase increased to 100% as the temperature increased up to 65°C (Fig. 1). Maximum activity was obtained at 65°C and pH 7.0 for the substrate starch ($20gL^{-1}$). Above 65°C, amylase activity was decreased sharply due to thermal denaturation of the enzyme and lost the activity. Hence 65°C was chosen as the optimum temperature for the assay of amylases. The purified α-amylase of *Bacillus licheniformis* CUMC 305 showed maximal activity at 90°C and pH 9.0 [17].

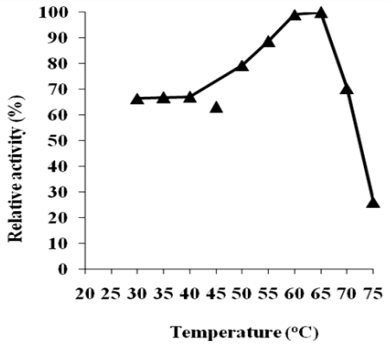

Fig. 1: Effect of temperature on the activity of amylase from fermented sweet sap of palmyrah with starch ($20gL^{-1}$) at pH 7.0. Amylases activities were measured at different temperatures using $20gL^{-1}$ starch as substrate by incubating for 5min at pH 7.0.

3.3 Effect of pH on the activity of amylases

When the pH was increased, the maximum activity of amylases was obtained at pH 7.0 (Fig. 2). The dependence of enzyme activity on pH is a consequence of the amphoteric properties of proteins [18].

3.4 Substrate specificity of the amylases

Different substrates were hydrolyzed by amylase enzyme. When 20gL^{-1} of lactose, maltose, pectin and sucrose were used as substrates to amylase enzyme that showed 168, 138, 74 and 55% of relative activity (Table 1) when compared to soluble starch at 65^{o}C and pH 7.0.

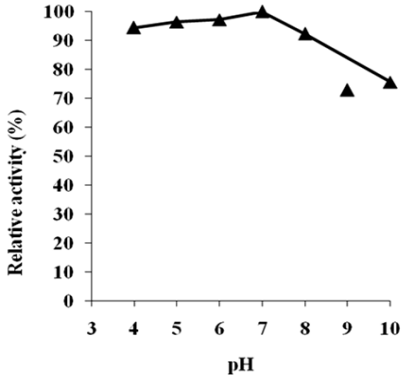

Fig. 2: Effect of pH on the activity of amylase from fermented sweet sap of palmyrah with starch (20gL^{-1}) at pH 65^{o}C. Activities were measured at different pH, using 20gL^{-1} starch as substrate by incubating for at 5minutes at 65^{o}C.

Table 1: Effect of different carbon sources on the activity of amylase from fermented sweet sap of palmyrah. Amylase activity was determined at 65^{o}C and pH 7.0 using 20gL^{-1} different substrate by incubating for at 5min.

Substrate (20gL^{-1})	Relative amylase activity (%)
Starch	100
Lactose	168
Maltose	138
Pectin	74
Sucrose	55

The relative rates of hydrolysis of amylase, soluble starch, amylopectin and dextrin by α-amylase from *Bacteroides amlophilus* were 100, 97, 92 and 60% respectively [19]. Krishnan *et al* [17] showed that the substrate specificity of purified Alpha-amylase from *Bacillus licheniformis* CUMC305 with different 1% substrates, release of reducing sugar were very rapid from amylase (129%) but was slower from soluble starch (101.4%), amylopectin (58.3%) and glycogen (100%). Alpha-amylase from *Bacillus licheniformis* ATCC 6346, exhibited no activity with pullulan and hydrolysis of amylose, starch and amylopectin were 119.3, 100 and 77.7% respectively [20]. Starch, amylase and amylopctin were the substrates preferentially hydrolysed by a-amylase from *Aspergillus tamari* [21].

3.5 Effect of temperature on the stability of amylase

When the amylase enzyme was pre-incubated at 65^{o}C and pH 7, 97 % of its initial activity was retained for 10 min and lost 62 % of its original activity at 60 min (Fig.3).

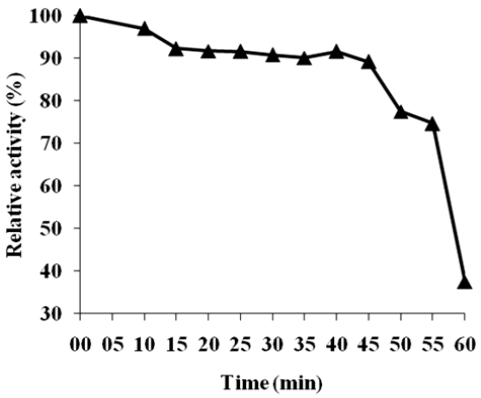

Fig. 3. Effect of of Temperature on the stability of amylase from fermented sweet sap of palmyrah. Amylase activity was measured at 65^{o}C using 20 gL^{-1} starch as substrate and incubating for 5 min pH at 7.0.

3.6 Effect of cations on the activity and stability of amylase

The effect of 2 mM Ca^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Na^{+}, Hg^{2+}, Zn^{2+}, Mn^{2+} and Cu^{2+} on amylase activity are presented in Table 1. In the presence of Mn^{2+}, Cu^{2+}, Ba^{2+}, Zn^{2+} and Na^{+}, the enzyme showed higher activity at 65^{o}C and pH 7.0. A slight increase in enzyme activity was produced by Mg^{2+} and stronger inhibition by Hg^{2+} (Table 2). Amylase of soybean seeds exhibited marked activating effects on the activity in the presence of 2mM Cobalt (II) and Manganese (II), enhancing up to 200% of the initial activity while Mercury (II) ions severely inhibited, however K^{+}, Ca^{2+}, Mg^{2+}, Al^{3+}, CU^{2+}, Zn^{2+} and Fe^{3+} moderately increased the enzyme activity to a certain extent [22]. Presence of Mn^{2+} and Fe^{2+} enhanced the activity of alpha-amylase produced by *Aspergillus oryzae* and is almost doubled in presence of Mn^{2+} [23] and activity of α-amylase from *Bacillus licheniformis* ATCC 6346 was strongly inhibited by Cu^{2+}, $Hg2^{+}$ and Mn^{2+} but less affected by Mg^{2+} and Ba^{2+}. Ca^{2+} and Na^{+} stimulated the enzyme activity at 85 ^{o}C and at pH 7.0 [24].

Table 2: Effect of different cations (2 mM) on the activity of amylase from fermented sweet sap of palmyrah. Amylase activity was determined at 65^{o}C and pH 7.0 using 20gL^{-1} starch as substrate by incubating for at 5min.

Cations (2 mM)	Relative enzyme activity (%)
Control	100
Ca^{2+}	136
Ba^{2+}	124
Mg^{2+}	119
Na^{+}	139
Hg^{2+}	003
Cu^{2+}	139
Zn^{2+}	136
Mn^{2+}	149

When amylase containing 2mM of Mn^{2+} Cu^{2+} and Na^{+} was incubated separately at pH 7.0 and 65^{o}C, amylase showed a higher stability in the presence of Cu^{2+} than control and the presence of other two ions (Fig. 4). When amylase was pre-incubated in the presence of 2mM of Mn^{2+} Cu^{2+} and Na^{+} seperately, the enzyme activity was retained at 79, 86 and 68 % respectively at 60 min but in the absence of cations (Control) enzyme retained 38% of its initial activity at 60min.

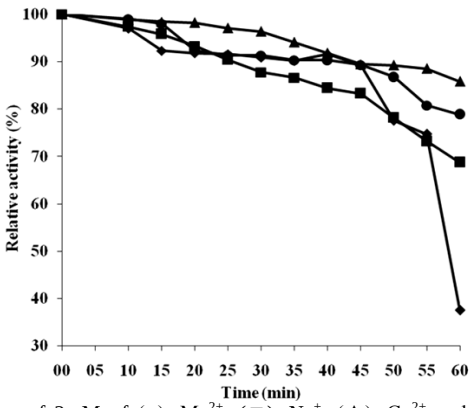

Fig. 4. Effect of 2mM of (●), Mn^{2+}; (■), Na^{+}; (▲), Cu^{2+} and (♦), control (without addition of any ions) on the stability of amylase from fermented sweet sap of palmyrah at 65°C. Amylase activity was measured at 65°C using 20 gL^{-1} starch as substrate and incubating for 5 min pH at 7.0.

4. CONCLUSION

The amylase enzyme produced by the activity of yeast cells during natural fermentation of sweet sap of palmyrah palms. Presence of Ca^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Na^{+}, Zn^{2+}, Mn^{2+} and Cu^{2+} increases the activity and stability of the amylase enzyme.

5. ACKNOWLEDGMENT

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