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Raw Starch-Digesting Amylase from *Saccharomycopsis fibuligera* 2074 Isolated from *Bubod* Starter

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Eight microbial isolates from *bubod* starter purchased from the Philippine National Collection of Microorganisms displayed amylolytic activity on raw sago starch indicating that they are possible sources of raw starch-digesting amylases (RSDA). *Saccharomycopsis fibuligera* 2074 showed the highest activity followed by *Saccharomycopsis bubodii* 2066 as determined through Dinitrosalicylic Acid Method. For *Saccharomycopsis fibuligera* 2074, maximum amylase production was obtained from 36-hour culture using 1% raw sago starch as carbon source under static incubation. The enzyme was purified via two-step purification protocol involving ammonium sulfate precipitation and diethylaminoethyl cellulose chromatography to give a specific activity of 180.49 U/mg and 2.57 purification fold. Further characterization of the enzyme showed that the amylase activity was optimum at pH 6 and temperature of 40°C. Although the enzyme was inhibited by Cu^{2+}, Zn^{2+}, and Al^{3+}, it was activated by Ca^{2+}, Fe^{3+}, Ba^{2+}, phenylmethylsulfonyl fluoride and ethylenediaminetetraacetic acid. On the other hand, iodoacetic acid, K^{+}, Cd^{2+}, and Mg^{2+}, showed no significant effect on the amylase activity. *Saccharomycopsis fibuligera* 2074 showed to be a promising source of RSDA to allow the direct and less costly conversion of raw sago starch to glucose.

Key words: amylolytic activity, *Bubod* starter, RSDA, *Saccharomycopsis fibuligera,* sago starch

INTRODUCTION

In the Philippines particularly in the Mindanao region, sago palm (*Metroxylon sagu* Rottb.) is an abundant indigenous source of raw starch (Flores 2008). However, utilization of sago starch in food is yet very limited as Filipinos prefer rice or corn as staple. An alternative promising use of sago starch is for the production of glucose which in turn, can be converted to many high-value products such as ethanol and lactic acid. However, due to the resiliency of starch structure, it needs to undergo pre-treatment steps consisting of gelatinization, liquefaction and saccharification prior to its complete hydrolysis to glucose. These processes require high energy input as they entail heating of starch at elevated temperatures for relatively long time. This renders production of starch hydrolysates very expensive (Mamo and Gessesse 1999; Goyal et al. 2005).

A promising strategy that will serve to bypass the energy-costly pre-treatment steps in starch processing is the use of raw starch-digesting amylases (RSDA). These are enzymes that can directly hydrolyze raw starch to glucose, hence, can definitely lower the production cost of high-value products from starch hydrolysates.

It has been reported that several microbial species display RSDA activity. Hamilton et al. (1999) reported that

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Bacillus sp. IMD 434 produced a raw starch-digesting amylase which was capable of hydrolyzing both soluble starch and raw corn starch to release glucose and maltose. Goyal et al. (2005) was able to isolate a thermostable alpha-amylase from a novel strain of *Bacillus* sp. I-3 obtained from natural soil samples which showed high raw starch-digesting activity towards potato starch. Two amylases designated as AmyI and AmyII were also isolated, purified and characterized by Mamo and Gessesse (1999) from *Bacillus* sp. Strain WN 11. Many fungi are also capable of producing raw starch-digesting amylases. These include *Aureobasidium pullulans* N13d (Li et al. 2007), *Aspergillus oryzae* (Yu et al. 1999) and *Aspergillus awamori* (Matsubara et al. 2004).

The fermentation starter for rice wine, locally known as *bubod*, has also been reported to show traces of raw starch-digesting amylases which are either secreted or contained in amylolytic microorganisms (Hesseltine et al. 1988). Sakai and Caldo (1985) isolated 560 yeasts from eight *bubod* samples, and found that the predominant genus was the amylase-producing *Endomycopsis* spp. (now known as *Saccharomycopsis* spp.) which comprised 45.5% of the isolates. Although their study reported on the amylolytic activity of *bubod* isolates based on Lugol's Test, no further elucidation of their biochemical characteristics was conducted. It is on this premise, coupled with the scientific and economic value of raw starch-digesting amylases that this study was conducted.

Pure cultures of eight *bubod* isolates namely, *Saccharomycopsis fibuligera* 2074 isolated from Agoo, La Union; *Saccharomycopsis fibuligera* 2076 from Banaue, Mountain Province; *Saccharomycopsis fibuligera* 2081 from Tublay, Mountain Province; *Saccharomycopsis bubudii* 2061 from Nueva Ecija; *Saccharomycopsis bubudii* 2066 from Tublay, Mountain Province; *Saccharomycopsis butonii* 2072 from Mainit, Mountain Province; *Hansenula anomala* 2063 from Sagada, Mountain Province; and *Saccharomycopsis capsularis* 2105 from Baguio City were purchased from the Philippine National Collection of Microorganisms (PNCM) at BIOTECH, University of the Philippines, Los Banos, Laguna, Philippines for use in this study. This research was conducted to (a) compare the RSDA activity of the eight *bubod* isolates; (b) improve the RSDA production of the superior isolate; and (c) purify and partially characterize the RSDA from the superior isolate.

MATERIALS AND METHODS

Bubod Microorganisms

Pure cultures of *Saccharomycopsis capsularis* 2105, *Saccharomycopsis fibuligera* 2081, *Saccharomycopsis fibuligera* 2074, *Saccharomycopsis fibuligera* 2076, *Saccharomycopsis bubodii* 2061, *Saccharomycopsis bubodii* 2066, *Saccharomycopsis bubodii* 2072 and *H. anomala* 2063, procured from PNCM were cultured in modified Yeast Malt Peptone (mYMP) medium (Dizon and Capareda, 2002) composed of 0.3% yeast extract (Laboratorios Conda S.A.), 0.3% malt extract (Difco Laboratories), 0.5% peptone (HiMedia Laboratories Rvt. Ltd.) and 1% sago starch. They were maintained in mYMP agar slants or plates, sub-cultured every month at 30°C without agitation for 24 h and kept refrigerated until further use. All media were sterilized by autoclaving at 121°C for 15 min at a pressure of 15 lb per square inch.

Production of Amylase

A volume of 1.8 mL mYMP contained in a test tube was inoculated with a loopful of the stock culture. It was incubated for 24 h at 30°C without agitation. This served as the starter inoculum for amylase production. It was added to 10.2 mL of mYMP medium and subsequently incubated for 24 h at 30°C. The amylase produced was harvested by centrifugation of the broth at 4°C at a speed of 10,000 x g for 10 min using Techcomp CT15RT Refrigerated Centrifuge. This served as the crude cell-free extract (CFE) which was used for the estimation of amylase activity.

Assay for Amylase Activity

The amylase activity was measured by quantifying the amount of reducing sugar produced through the DNS (Dinitrosalicylic Acid) method with slight modification (Borel et al., 1952). Fifty microliters of diluted enzyme solution (1:15 dilution) was added to 500 µL of substrate (1% soluble potato starch in 0.1 M acetate buffer) in a test tube. The reaction was carried out at 30°C for 10 min, after which, 1 mL of DNS reagent was added to stop the reaction. The test tube was covered with marble and heated in a boiling water bath for 10 min. It was cooled in running water to room temperature and 5.0 mL distilled water was added. The reducing sugar released from the hydrolysis of starch was quantified by measuring the absorbance through a UV-VIS Spectrophotometer (UV-1610A PharmaSpec, Shimadzu, Japan) at a wavelength of 500 nm using glucose as standard. One unit of enzyme activity for amylase is defined as the amount of enzyme required to produce 1 µmol of glucose per min under assay conditions. All assay analyses were done in triplicate.

Protein Content Determination

Protein concentration was estimated through the Bradford method (Bradford, 1976). In a test tube containing 60 µL of protein sample, 3 mL of Bradford dye reagent

was added. The solution was thoroughly mixed and allowed to stand at room temperature for 5 min. The absorbance of the solution was measured using a UV/VIS spectrophotometer (UV-1610A PharmaSpec, Shimadzu, Japan) at a wavelength of 595 nm using bovine serum albumin (BSA) as standard.

Optimization of Amylase Production

The production of amylase was optimized by monitoring microbial growth and amylase activity at varying fermentation time, modifying the type of starch preparation used in the fermentation medium, and determining the effect of agitation during fermentation.

Effect of fermentation time

Amylase production was carried out at varying fermentation time up to 96 h. For the first 24 h, amylase activity was monitored at three-hour interval through the DNS method described above. Subsequently, monitoring was done on the 48th and 96th hours. Microbial pour plating was also performed to monitor yeast count at each time interval in order to correlate microbial growth with enzyme activity and fermentation time.

Effect of starch gelatinization

Two types of starch preparation used in the fermentation medium were tested in this study, namely, raw and gelatinized sago starch. For the set-up involving gelatinized starch, gelatinization was achieved when the medium was sterilized by autoclaving at 121°C for 15 min at a pressure of 15 lb per square inch. On the other hand, for the setup involving raw sago starch, the starch was excluded from the medium during autoclaving. Rather, it was dry-sterilized separately in a convection oven at 180°C for 3 h. The sterile broth was then added aseptically to the sago starch to achieve a concentration of 1% starch. Production of amylase and subsequent assay were done following the protocols described above.

Effect of agitation

The effect of agitation was studied in order to determine whether oxygen can affect the production of amylase. The first setup involved production of the enzyme under static condition for 24 h at 30°C. The second setup involved production of the enzyme with agitation at a speed of 100 revolutions per minute (rpm). Assay for amylase activity was then done following the DNS method described above.

Purification of the Enzyme

Enzyme purification was achieved following a two-step purification protocol involving ammonium sulfate precipitation followed by dialysis, and anion-exchange chromatography.

Ammonium sulfate precipitation and dialysis

Optimization of ammonium sulfate concentration was performed prior to dialysis. Initial purification step of amylase was carried out by precipitating the enzyme from the supernatant with addition of solid ammonium sulfate from 30% to 90% (w/v) saturation based on the method of Krishnan and Chandra (1983). The concentration of ammonium sulfate which showed the highest amylase activity was chosen as the optimum condition. After addition and dissolution of the salt, the solution was aged overnight to facilitate complete precipitation of the target amylase. The reaction mixture was then centrifuged at a speed of 4,500 x g for 30 min at 4°C to separate the precipitate. The supernatant was discarded while the precipitate was redissolved in 30 mL of 0.1 M Tris-HCl buffer (pH 9.0). The dissolved precipitate was poured into a dialysis tubing of 30-kDa molecular weight cut-off (MWCO). The bag was then sealed, attached onto a glass rod and suspended in 0.1 M Tris-HCl buffer (pH 9) for 24 h under refrigerated temperature. The dialyzing buffer was changed regularly during the whole duration of the dialysis. After dialysis, the bag was cut open, after which, the dialysate volume was measured, and the amylase activity was quantified through the DNS method described above.

DEAE-cellulose column chromatography

The final purification step was performed through anion-exchange chromatography using diethylaminoethyl (DEAE) cellulose column following the method of Nguyen et al. (2002) with modifications. The dialysate was loaded to a column (2.5 x 50 cm) pre-equilibrated at room temperature (~28°C) in 0.1 M Tris-HCl buffer (pH 9.0). A fraction collector equilibrated at a flow rate of 3 mL/49 s was used to gather all the fractions eluted. Using NaCl gradient (0-1.0 M), proteins were eluted stepwise in the same buffer. The absorbance of each fraction was read in spectrophotometer at a wavelength of 280 nm (A_{280}). Peak fractions were tested for amylase activity. The pooled fraction with the highest activity was used in the succeeding procedures.

Amylase Characterization

The DEAE column-purified enzyme was characterized by determining its optimum activity and stability under varied temperature and pH, as well as its optimum pH, optimum temperature, and reaction to several chemical modifiers based on the methods of Takeuchi et al. (2006) with some modifications.

Determination of Optimum pH

Determination of the optimum pH was done by adding 50 µL of enzyme solution (1:15 dilution) to 500 µL of substrate consisting of 1% soluble potato starch dissolved in each of the following buffers: 0.1 M glycine-HCl buffer (pH 3-3.5), 0.1 M acetate buffer (pH 4.0-5.5), 0.1 M phosphate-citrate buffer (pH 6.0-7.0), 0.1 M Tris-HCl buffer (pH 7.5– 9.0) and 0.1 M glycine-NaOH buffer (pH 9.5-10.5). The reaction was carried out at 30°C for 10 min. The amylase activity was then quantified following the DNS method described above. The pH condition that yielded the highest amylase activity was considered as the optimum pH of the enzyme.

Determination of Optimum Temperature

Determination of the optimum temperature was done by adding 50 µLof enzyme solution (1:15 dilution) to 500 µL of substrate consisting of 1% soluble potato starch dissolved in 0.1 M acetate buffer. The reaction was carried out at varying temperatures ranging from 30°C to 80 °C at 10°C increment for 10 min. The amylase activity was then quantified following the DNS method described above. The temperature condition that yielded the highest amylase activity was considered as the optimum temperature of the enzyme.

Effects of pH and temperature on amylase stability

The effect of pH on amylase stability was determined by pre-incubating the enzyme at different pH values ranging from 3.0 to 10.5 for 24 h. The amylase activity was then quantified following the DNS method described above.

The effect of temperature on the stability of the enzyme was performed by pre-heating the enzyme at temperatures ranging from 30°C to 80°C for 30 min. The amylase activity was then quantified following the DNS method described above.

Effects of chemical modifiers on amylase stability

The influence of chemical modifiers on amylase stability was determined by pre-incubation of the DEAE column--purified enzyme at 40°C for 15 min with different metal ions ($CaCl_2$, KCl, $CuCl_2$, $BaCl_2$, $AlCl_3$, $ZnCl_2$, $MgCl_2$, $CdCl_2$, $FeCl_3$) and organic reagents namely, ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF) and iodoacetic acid (CH_2ICOOH) making the final concentration of the chemical modifier in the enzyme solution to be equal to 10 mM. The amylase activity was then quantified following the DNS method described above.

Statistical Analysis

All experiments were carried out using three trials, with three replicates each. The samples collected from each replicate were analyzed using one-way Analysis of Variance (ANOVA): Post Hoc Multiple Comparison Test. Means of amylase activity at each factor were calculated and significant differences were determined along with their standard errors.

RESULTS AND DISCUSSION

Comparison of the amylolytic activity of isolates from *Bubod* starter

Quantitative determination of the amylolytic activity of the eight *bubod* isolates was done through the Dinitrosalicylic acid (DNS) method based on the amount of reducing sugar produced. Results indicated that all eight *Bubod* strains could act on raw sago starch, hence, they are potential sources of RSDA. Among the strains, *Saccharomycopsis fibuligera* 2074 significantly manifested the highest amylase activity while *Saccharomycopsis bubudii* 2061 and *H. anomala* 2063 showed the lowest amylolytic activity (Figure 1). No significant differences in the amylolytic activity were observed among *Saccharomycopsis fibuligera* 2076, *Saccharomycopsis bubudii* 2066, and *Saccharomycopsis fibuligera* 2081, as well as between *Saccharomycopsis capsularis* 2105 and *Saccharomycopsis burtonii 2072*. Since *Saccharomycopsis fibuligera* 2074 showed to be superior in terms of RSDA activity, it was selected for further investigation.

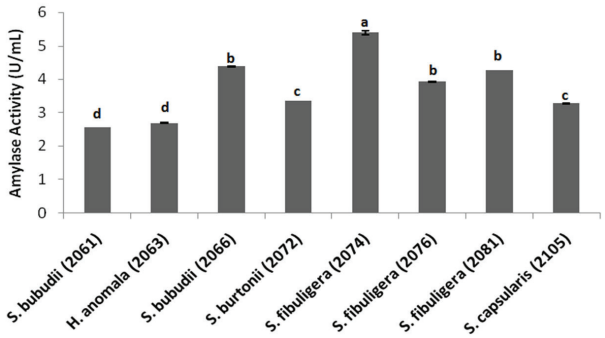

Figure 1. Amylolytic activity of Bubod isolates using raw sago starch as substrate.

Optimization of Amylase Production

Studies on the effect of fermentation time on the production of amylase from *Saccharomycopsis fibuligera* 2074 showed that the specific activity of the enzyme peaked at 36-h incubation. This behaviour correlated well with results obtained on the growth profile of the yeast whereby cell mass also peaked after 36 h (Figure

2) as determined through colony count using pour plating method. Enzyme activity and cell growth rapidly decreased after 36 h. The decline of enzyme activity after the 36th h may be due to one or all of the following: (a) nutrient depletion (b) competition among microorganisms and (c) rise of protease levels in the environment (Hiller et al. 1996; Soni et al. 1996; Swamy and Seenayya 1996).

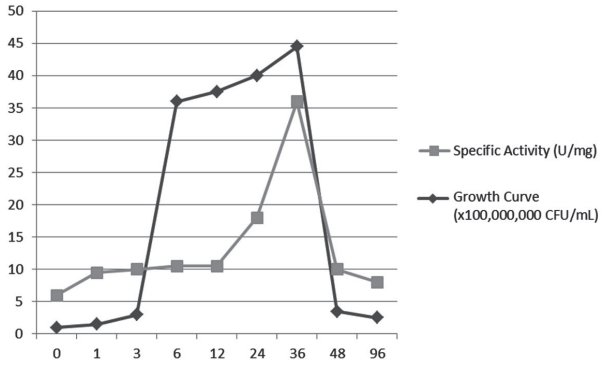

Figure 2. Time course study for growth and amylase production of *Saccharomycopsis fibuligera* 2074.

Inducing the microorganism to over-produce the enzyme of interest is the best way of obtaining higher yield (Rosenberg 1996). This can be achieved by controlling the physical environmental factors or the components of the growth medium. *Saccharomycopsis fibuligera* 2074 was grown on modified YMP, where sago starch (1%) served as carbon source. Goyal et al. (2005) noted that the form of starch by which it is made available to the fungi affects its amylase production. Figure 3 shows that the culture supplemented with raw sago starch resulted in specific activity of amylase which is two-fold greater than that supplemented with gelatinized sago starch. This is a strong indication that *Saccharomycopsis fibuligera* 2074 produces raw starch-digesting amylase (RSDA).

The oxygen transfer in a shake flask is usually a critical factor in the production of amylase (El Enshashy 2007; Clark et al. 1995). Agitation or shaking can be a form of oxygen transfer. Results showed that the fermentation medium of *Saccharomycopsis fibuligera* 2074 for amylase production from raw sago starch should not be agitated since shaking caused significant reduction in amylase activity (Figure 4). This decrease may be due to mechanical stress and physical damage to the culture medium upon agitation. It has been known that *Saccharomycopsis fibuligera* 2074 is a facultative anaerobe, therefore, it can still survive in relatively low levels of oxygen (Hostinova 2002).

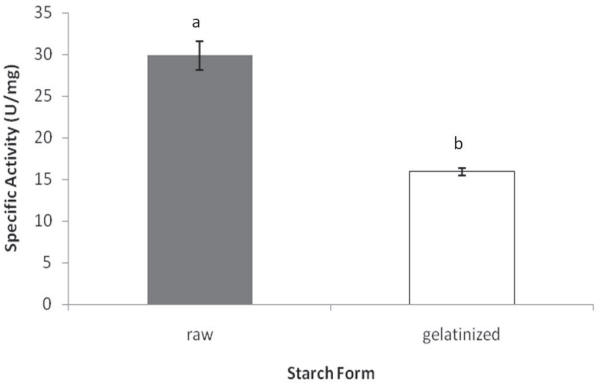

Figure 3. Effect of sago starch form on the specific activity of *Saccharomycopsis fibuligera* 2074.

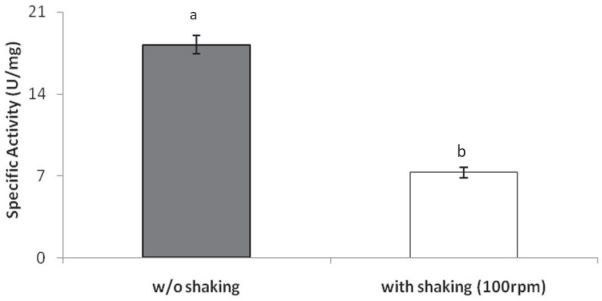

Figure 4. Effect of agitation on the specific activity of *Saccharomycopsis fibuligera* 2074.

Purification of Amylase

The purification of the enzyme improves its amylolytic activity and relatively lengthens enzyme potency, as compared to the crude preparations with lower enzymatic values and high impurity content (Takasaki 1976). One of the most commonly used techniques to purify protein is by precipitation using ammonium sulphate. Purification studies on *Bacillus* sp. A721, *Bacillus* sp. strain WN11, *Bacillus subtilis* AX20, *Streptococcus bovis* JB1 and *Thermomyces lanuginosus* strain ATCC 34626 used this method as preliminary purification technique implying its popularity (Freer 1993; Mamo and Gessesse 1999; Maitin et al. 2001; Nguyen et al. 2002; Najafi et al. 2005). In this study, crude protein preparations were precipitated with solid ammonium sulfate. Optimization of the percent saturation of the salt was done in order to determine the exact amount that would result in maximum amylase activity.

Results revealed that the enzyme from *Saccharomycopsis fibuligera* 2074 started to precipitate at 60% saturation yielding a specific activity of 43.10 U/mg (Figure 5). Increasing the percent saturation did not significantly increase amylase activity, rather, activity levelled off as it approached 80% saturation. Thereafter, amylase activity

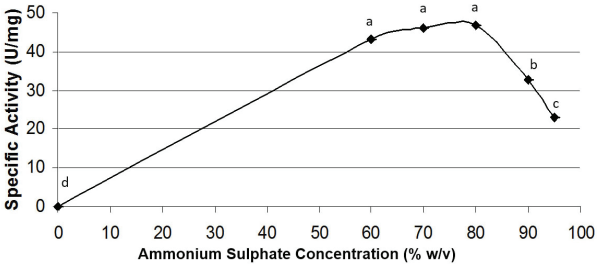

Figure 5. Optimized concentration of ammonium sulfate for precipitation of amylase from *Saccharomycopsis fibuligera* 2074.

rapidly decreased suggesting that small amounts of proteins were left after precipitation. Since 60% saturation showed amylase activity that was statistically equal with those at 70% and 80% saturation, it can then be inferred that ammonium sulfate precipitation is best performed at 60% saturation. It is more economical considering the purification cost.

Table 1 shows the result of the purification protocol performed for *Saccharomycopsis fibuligera* 2074. After ammonium sulphate precipitation and dialysis, the enzyme was purified 1.36-fold which is higher than those obtained by other researchers on several microbial amylases (Table 2). Further purification through anion-exchange chromatography using DEAE-cellulose column yielded a purification fold of 2.57 which is comparable to that obtained by Takeuchi et al. (2006) for the alpha-amylase from *Pichia burtonii* with a purification fold of 2.95. On the other hand, this result is essentially higher than those obtained by other enzymologists who also used diethylaminoethyl (DEAE)-cellulose column chromatography as a purification step for amylases (Table 3). The purification of the RSDA from *Saccharomycopsis fibuligera* 2074 was successful as evidenced by the increasing purification fold and specific activity of the DEAE column-purified enzyme relative to the crude extract as reflected in Table 1.

Table 2. Comparison of enzyme purification fold achieved after dialysis.

Microbial Source	Purification Fold	Reference
Saccharomycopsis fibuligera 2074	1.36	This study
Lactobacillus manihotivorans LMG10810^T	1.36	Aguilar et al. 2000
Thermomyces lanuginosus strain ATCC 34626	1.20	Nguyen et al. 2002
Bacillus subtilis AX20	1.10	Najafi et al. 2005
Aureobasidium pullulans N13d	1.10	Li et al. 2007

Table 3. Comparison of enzyme purification fold achieved after DEAE-cellulose chromatography.

Microbial Source	Purification Fold	Reference
Saccharomycopsis fibuligera 2074	2.57	This study
Pichia burtonii 15-1	2.95	Takeuchi et al. 2006
Aspergillus oryzae NR3-6	2.30	Yu et al. 1999
Thermomyces lanuginosus strain ATCC 34626	1.60	Nguyen et al. 2002

Characterization of DEAE column-purified amylase from *S. fibuligera* 2074

Optimum pH and temperature of amylase

Temperature and pH primarily determine the use of amylase in industrial operations. The influence of pH on enzyme activity was examined from pH 3 to 10.5 for 10 min (Figure 6). The amylase from *Saccharomycopsis fibuligera* 2074 showed optimum pH at 6. A decline in amylase activity was recorded below and above pH 6.

Study on the effect of temperature on amylase activity was performed from 30°C to 80°C. The results showed that the amylase from *Saccharomycopsis fibuligera* achieved highest

Table 1. Purification of amylase from *Saccharomycopsis fibuligera* 2074.

Purification Steps	Volume (mL)	Activity (U/mL)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification fold	Yield (%)
Culture supernatant (crude extract)	250	9.11	2,277.5	32.25	70.34	1.00	100
Ammonium sulfate precipitation and dialysis	44	8.12	357. 28	3.74	95.50	1.36	15.69
Ion-exchange chromatography: DEAE-cellulose	39	5.71	222.69	1.23	180.49	2.57	9.78

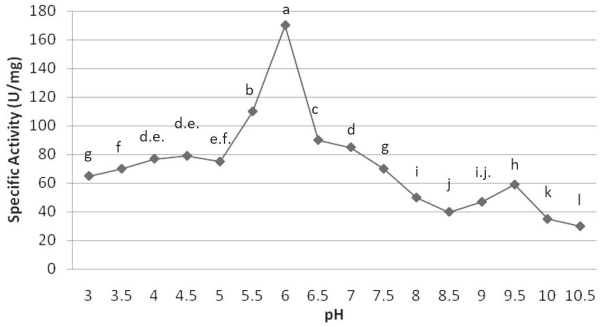

Figure 6. Optimum pH of *Saccharomycopsis fibuligera* 2074 amylase.

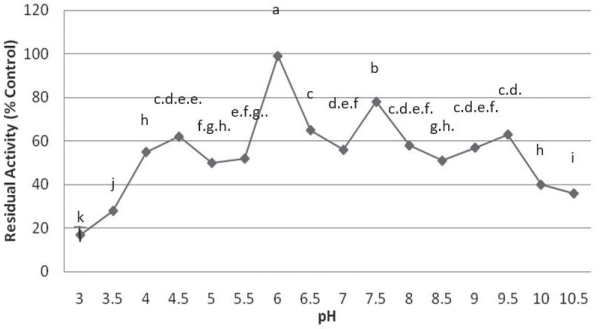

Figure 8. Stability of *Saccharomycopsis fibuligera* 2074 amylase on various pH values.

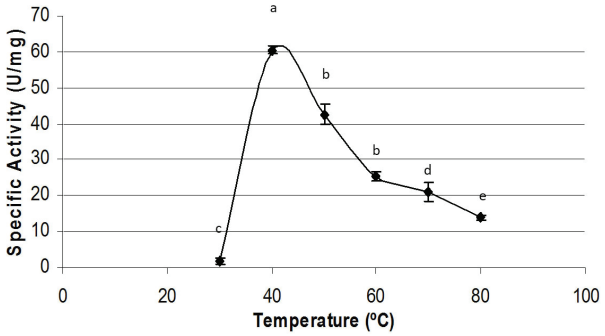

Figure 7. Optimum temperature of *Saccharomycopsis fibuligera* 2074 amylase.

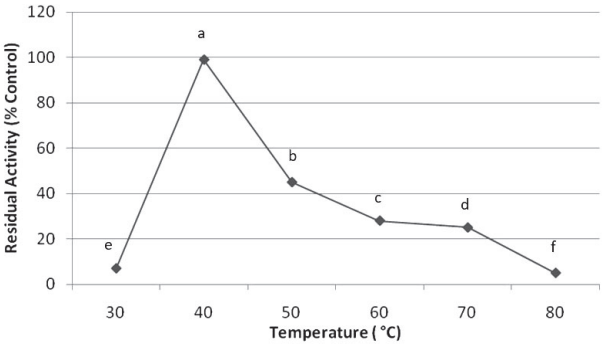

Figure 9. Stability of *Saccharomycopsis fibuligera* 2074 amylase on various temperatures.

hydrolyzing activity at 40°C (Figure 7). It was followed by a gradual decrease in amylase activity as the temperature increased. Low specific activity was observed at 30°C because generally at low temperature, reaction rates are slow.

Stability of purified amylase on various pH and temperatures

To further characterize the DEAE column-purified amylase from *Saccharomycopsis fibuligera* 2074, the stability of the enzyme at different pH values were evaluated under pH range of 3 to 10.5 (Figure 8). Results showed that the enzyme was found significantly stable at pH 6. It was able to retain 99.99% of its amylase activity. The pH stability profile showed that the activity of *Saccharomycopsis fibuligera* 2074 amylase concomitantly declined at extreme pH values. This can be attributed to denaturation of the amylase.

Response of *Saccharomycopsis fibuligera* 2074 amylase on varied temperature is shown in Figure 9. The enzyme showed good stability at a temperature of 40°C retaining 100% amylase activity. Results revealed that beyond 40°C, enzyme activities dramatically dropped. At 80°C, only 4% of the activity remained. These observations are in agreement to the general stability trend of yeast and mold amylases which are not naturally thermostable except if they have undergone genetic modification (Barbier 1997).

Effect of chemical modifiers on amylase stability

Maximum catalytic activity may be achieved by supplementation of metal ions as they are responsible for the right orientation of active site of amylases. The folded state of protein can be stabilized by metal binding during which metal ions are coordinated usually by lone pair donation from oxygen or nitrogen atoms (Bhatti et al. 2005). The amylase activity of *Saccharomycopsis fibuligera* significantly increased in the presence of calcium chloride and ferric chloride by 233% and 164%, respectively (Figure 10). Therefore, amylase assay should be performed by supplementation of ample concentration of $CaCl_2$ or ferric chloride to enhance the amylase activity. However, the presence of divalent and trivalent metal ion such as Zn^{2+} and Al^{3+} respectively, reduced the amylase activity. Furthermore, Cu^{2+} completely inhibited the activity of the enzyme.

The effect of organic reagents on enzyme activity was also studied using PMSF, EDTA and iodoacetic acid. It was observed that EDTA and PMSF increased the amylase activity by 101% and 170%, respectively. Iodoacetic acid on the other hand, did not significantly affect the amylase activity.

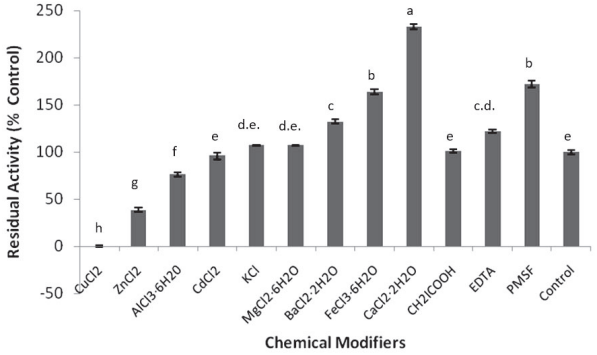

Figure 10. Stability of *Saccharomycopsis fibuligera* 2074 amylase as affected by various chemical modifiers.

SUMMARY AND CONCLUSION

Raw starch-digesting amylases (RSDA) are enzymes that offer a promising alternative to the conventional energy-costly hydrolysis of starch. The latter is a good resource for the production of glucose which can eventually be converted to other high-value products like ethanol and lactic acid. RSDA from *Saccharomycopsis fibuligera* 2074 obtained from *bubod* starter was isolated, purified and characterized. Results showed that the enzyme displayed optimum amylolytic activity when incubated in raw sago starch for 36 h without agitation. It was subsequently purified through ammonium sulfate precipitation and diethylaminoethyl chromatography to give a specific activity of 180.49 U/mg and 2.57 purification fold. The RSDA has an optimum pH of 6 and optimum temperature of 40°C. It was inhibited by Cu^{2+}, Zn^{2+}, and Al^{3+} but activated by Ca^{2+}, Fe^{3+}, Ba^{2+}, phenylmethylsulfonyl fluoride and ethylenediaminetetraacetic acid. Since the amylase activity of the partially-purified *Saccharomycopsis fibuligera* 2074 showed to be higher than those obtained from different sources by other researchers, it can be concluded that *Saccharomycopsis fibuligera* 2074 is a novel source of RSDA which can be further pursued in future studies for gene cloning and expression in *Saccharomyces cerevisiae* to allow the direct conversion of sago starch to ethanol.

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