POTENTIAL APPLICATION OF WASTEWATER FROM RICE NOODLE MANUFACTURE IN α -AMYLASE PRODUCTION

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Abstract

The feasibility of using wastewater from rice noodle manufacture as a substrate for the production of α-amylase was evaluated. The maximum α-amylase production by *Aspergillus* sp. NU4 was achieved after 24 h. Supplementation of appropriate nitrogen sources and inorganic salts to the wastewater medium significantly enhanced enzyme production. The enzyme yield reached a maximum of 36.5 U/ml with the wastewater adding with 10 g/l defatted soybean, 10 g/l KH_2PO_4, 5 g/l $MgSO_4.7H_2O$, and 0.1 g/l $ZnCl_2$. It accounted for 8-fold increase. The α-amylase of *Aspergillus* sp. NU4 was subjected to catabolite repression. It was partially purified using 60% ammonium sulfate. The optimum pH and temperature of the partially purified enzyme were determined to be 5 and 50°C, respectively. After 2-h incubation, the enzyme degraded more than 90% of raw starch granules of potato starch, rice starch, and corn starch.

Keywords: Amylase, wastewater, rice noodle, *Aspergillus* sp.

Introduction

Rice noodle has been used for Thai cooking. The manufacture of noodle using rice starch as a raw material produces a large volume of wastewater. The wastewater causes a considerable disposal or treatment problem because of its high biological oxygen demand (BOD). Although treatment of the wastewater has been achieved by the method of activated sludge, from the point of view of resource recovery, this method is not economical. Therefore, development of an effective system for utilization of the wastewater is highly desirable. Through the level of total starch content was high in the wastewater, α-amylase production was selected as an appropriate target. Amylase producing fungi were isolated from the wastewater and selected for further investigation on the feasible production of α-amylase in a laboratory. The effects of environmental conditions, media formulation and characteristics of partial purified enzyme were then examined.

Materials and Methods

Microorganism and Fermentation Conditions

The amylase producing fungus used in this work was *Aspergillus* sp. NU4. It was obtained by isolation and screening from wastewater samples, collected from the local rice noodle manufacture in Phitsanulok during summer 2002. The fungus was grown on potato dextrose agar slants at 30°C for 5 days. Then, the bright

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green-spore slants were stored at 4°C until use. The organisms were subcultured every month. The spores were collected in 0.01% Tween-80 solution.

The fungal cultures were grown in 250-ml Erlenmeyer flasks containing 50 ml of wastewater under the following conditions unless otherwise stated. The initial pH of wastewater was 5.6 which was used without adjusting and the media were sterilized by autoclaving at 120°C for 20 min. Spore suspension (1 x 10^8 spores) was inoculated in the flasks which were then shaken on a rotary shaker at 250 rpm under room temperature (30 + 2°C). α-Amylase activity, protein content, and pH change were determined.

Analytical Procedures

α-Amylase activity was assayed by the method of McMahon *et al*. (1997) with slight modifications of optimum temperature and pH. One unit of α-amylase activity (U/ml) was defined as the activity of 1 ml of enzyme solution that produced 1 μmol of reducing sugar per minute from starch at pH 5 and 50°C. Reducing sugar was determined by the 3,5-dinitrosalicylic acid (DNS) method (Samarntarn and Tanticharoen, 1999) using glucose as a standard. The wastewater contained some suspended particles, which interfered with the determination of cell weight by filtration and drying. Thus, protein content was used in stead of dry mass for evaluation of cell growth. Protein contents were determined by the standard Kjeldahl method (Kjeldahl, 1883).

All the fermentation experiments and enzyme assay were carried out in triplicate with analytical grade reagents and the mean values were reported.

Properties of Partial Purified Enzyme

Optimum temperature was determined at various temperatures (35 - 60°C). Optimum pH was determined by measuring the activity in citrate buffer, KH_2PO_4-NaOH buffer, and boric acid-NaOH buffer (50 mM) at pH ranging from 3 - 6, 7 - 8 and 9 - 10, respectively. Thermal and pH stability of enzyme were examined by incubation of the enzyme at selected temperatures and pHs for up to 5 h with subsequent determination of the residual activity.

Hydrolysis of Raw Starches

The reaction mixture (5 ml), containing 25 mg (dry basis) starch granules, 50 mM sodium acetate buffer pH 5 and 2.5 U of enzyme, were incubated at 50°C with mild stirring to prevent starch from setting. Total reducing sugar was determined by the DNS method after hydrolysis with 25% HCl.

Results and Discussion

Isolation and Screening of Amylase Producing Fungi

Fourteen fungal strains were found to be able to produce amylase in the wastewater samples from rice noodle manufacture. During the preliminary screening using amylase production ability, the diameter of clear zones was determined. Among the strains examined, strain NU4 gave the highest amylase production and was chosen for further study of the feasible production of α-amylase by using the wastewater from the rice noodle manufacture. This strain was appeared to belong to the genera *Aspergillus* sp. according to the basis of its physiological and morphological characteristics.

Time Course Analysis of α-Amylase Production by *Aspergillus* sp. NU4

The production of α-amylase reached maximum activity of 4.5 U/ml at 24 h (Figure 1). The results indicated that the enzyme was secreted early in the active growth phase and reached maximum toward the end of the exponential growth phase. The enzyme activity significantly dropped after 24 h. It might be due to denaturation and/or decomposition of α-amylase as a result of interaction with other compounds in the fermented medium (Ramesh and Lonsane, 1987; Krishna and Chandrasekaran, 1996).

Effect of Starch Content of the Medium

It has been reported that the addition of starch at high concentration decreased the α-amylase yield (Iefuji *et al*., 1996; Krishna and Chandarasekaran, 1996). A high starch content medium, when attacked by α-amylase during

fermentation could have undergone degradation resulting in the accumulation of reducing sugar. It would have led to enhancement of sugar concentration of the substrate and catabolite repression of α-amylase synthesis. In this case, although the high starch content of wastewater caused thickening of medium broth, it did not lead to decrease α-amylase activity. To overcome an aeration problem, diluted medium was used. When the wastewater was diluted at the ratio up to 1 : 3, 2-fold enzyme reduction was observed (Figure 2). It might be due to the lower cell growth since the protein content of the medium was reduced 18% of the original (data not shown).

Effect of Initial pH

The results revealed a significant influence of pH of the medium used on the production of α-amylase by *Aspergillus* sp. NU4 (Figure 3). The production of α-amylase was more favorable at pH 5.0-8.0 at 24 hrs of incubation, which was about 7-fold higher than the maximum activity obtained at higher acidic or alkaline pH.

Enhancement of α-Amylase Production by the Addition of Nitrogen Sources and Inorganic Salts

The wastewater used in this study composed of high carbon source but low nitrogen

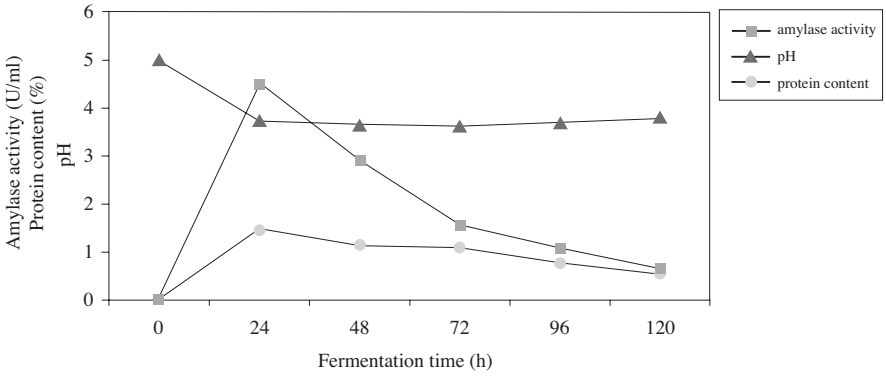

Figure 1. Fermentation profile of α-amylase production by *Aspergillus* sp. NU4 in wastewater medium at an initial pH of 5.6, 30 ± 2°C and 250 rpm.

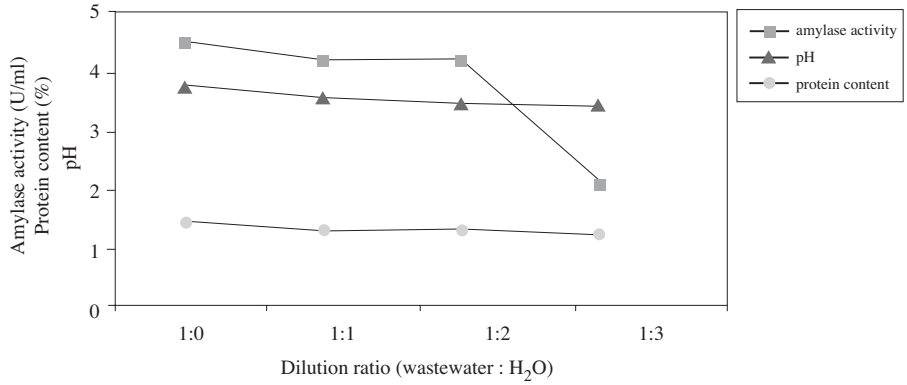

Figure 2. The effect of dilution ratio of wastewater as a sole substrate for α-amylase production by *Aspergillus sp*. NU4.

source. In an attempt to increase the α-amylase production, the effect of some nitrogen source, including $(NH_4)_2SO_4$, yeast extract and defatted soybean, was investigated. The results showed that defatted soybean, an agro-industry waste from oil manufacture, was the best nitrogen source to promote enzyme production (Figure 4). α-Amylase production increased with increasing defatted soybean up to 10 g/l. This might be due to the increase in growth yield at higher defatted soybean contents and further increase in total enzyme production. The amount of defatted soybean higher than 10 g/l could cause a decrease of α-amylase production by *Aspergillus* sp. NU4.

Ammonium sulfate was not a good nitrogen source for α-amylase production. It promoted α-amylase production less than two-fold at the concentration of 0.5%. It might be because ammonium sulphate lowered low pH of the medium from 5.6 to about 2.5. This caused the enzyme to be unstable. A similar increase in nitrogen source supplement was reported for banana fruit stalk (Krishna and Chandrasekaran, 1996) and sugarcane-press mud medium (Sandhya and Lonsane, 1994).

Some essential salts for cell growth also affected α-amylase production. When the salt mixture (10 g/l KH_2PO_4, 5 g/l $MgSO_4.7H_2O$, 0.1 g/l $ZnCl_2$) was added to the wastewater medium, α-amylase activity increased from 4.5 to 20.5 U/ml.

Effect of Glucose as a Catabolite Repressor

α-Amylase was reported to be inducible (Wind *et al*., 1994), constitutive (Forgaty *et al*., 1994) and may also be subjected to catabolite repression (Wind *et al*., 1994). The results indicated that α-amylase production by *Aspergillus* sp. NU4 was subjected to be catabolite repression. The catabolite repression was clearly seen when glucose (10 g/l) was added. The maximum enzyme yield decreased 27% (Figure 5). Catabolite repression of the biosynthesis of α-amylase poses serious problems of economy in the submerged fermentation process (Emanuilova and Toda, 1984). Consequently, fed-batch or continuous cultures and mutants resistant to catabolite repression need to be employed to partially or fully overcome the regulatory mechanism (Baig *et al*., 1984; Emanuilova and Toda, 1984; Saito and Yamamoto, 1975).

Partial Purification and Properties of the Enzyme

In order to examine the properties of the enzyme, the amylase from the culture broth was partially purified. A precipitation test was conducted using ammonium sulfate. After salting out with ammonium sulfate ranging from 30 to 80% saturation, recovery of α-amylase activity was below 50% under all test conditions. On the basis of these results, salting out with 60% ammonium sulfate was the most effective from the perspective of recovery and specific activity

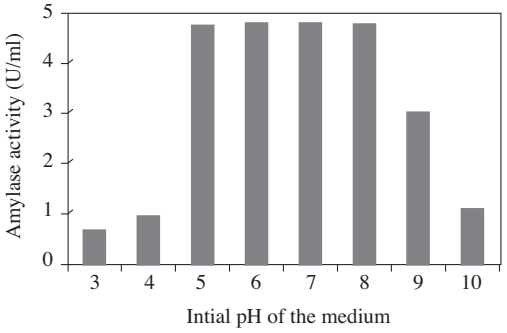

Figure 3. Effect of initial pH of wastewater medium on α-amylase production by *Aspergillus* sp. NU4.

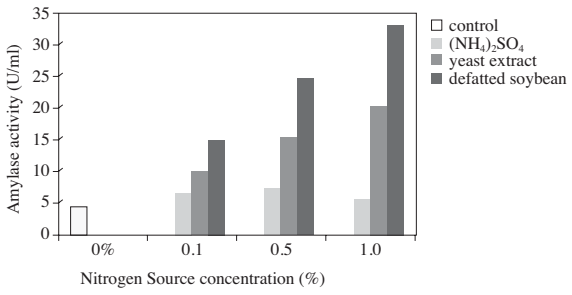

Figure 4. Effect of nitrogen sources supplemented in the wastewater medium on α-amylase production by *Aspergillus* sp. NU4.

of α-amylase (data not shown).

The enzyme was effective in the wide pH range with an optimum pH of 4 - 8 (Figure 6a). It could possibly be used commercially, since it can be operated in a wide range of pH of the substrate. The enzyme exhibited an optimum temperature of 50°C (Figure 6b). Remaining activity was 95, 85, and 58% at 45, 50 and 55°C, respectively, after 1 h of incubation (Figure 7). The α-amylase activities were completely lost after 5 h at 45, 50°C, and after 2 h at 55°C. It indicated that the enzyme was stable below 55°C, but became unstable above 55°C.

Hydrolysis of Raw Starches

Starch granules, in which molecules are densely packed in a polycrystalline state with inter- and intramolecular hydrogen bonds, are insoluble in water and often insensitive to chemicals and enzymes. When such starches are gelatinized by heating with water to improve their chemical reactivity for conversion with enzymes or microorganisms. In order to save the cost of gelatinization and to simplify the processes for conversion and fermentation, further study was made to investigate the capability of enzyme to degrade raw starch

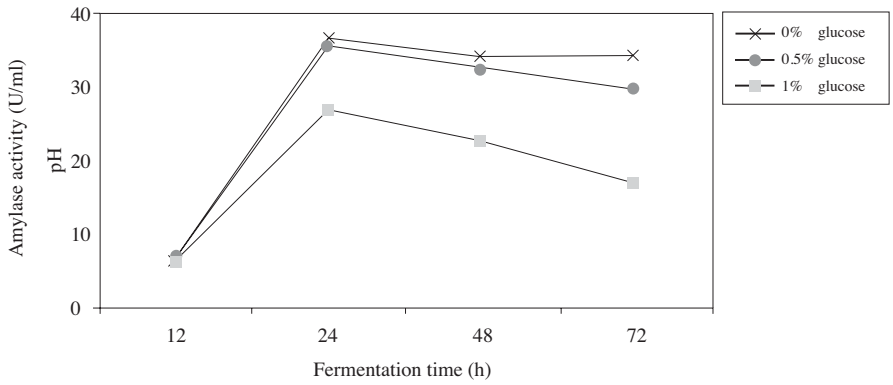

Figure 5. Effect pf glucose as a catabolite repressor on α-amylase production by *Aspergillus* sp. NU4.

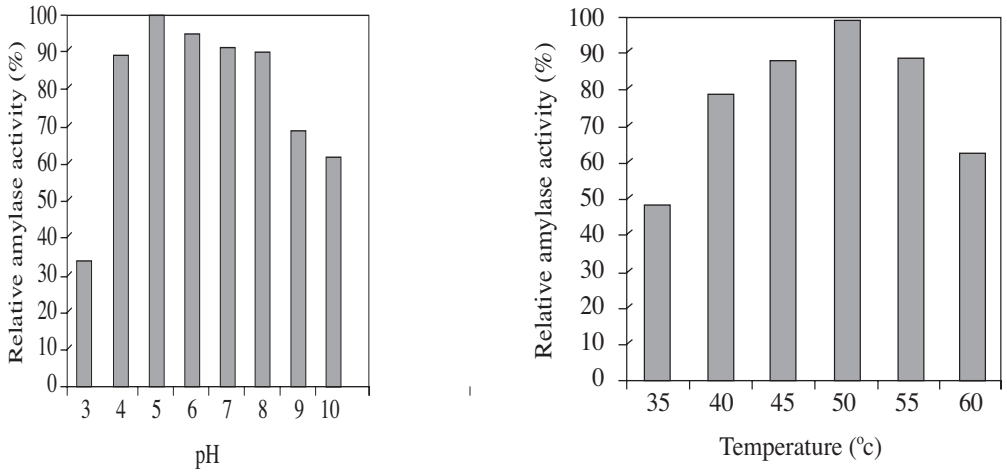

Figure 6. Optimum pH (a) and temperaure (b) of α-amylase of *Aspergillus* sp. NU4.

granules, including potato starch, rice starch, wheat starch and corn starch.

The extents of hydrolysis (as glucose) of raw potato starch, rice starch, wheat starch and corn starch, with α-amylase of *Aspergillus* sp. NU4 reached maximum of 100, 95.81, 48.97 and 96.26%, respectively, at 2 hrs incubation (Figure 8). It was inferred that prolonged incubation above 2 hrs at 50°C resulted in reduction of enzyme catalysis due to its thermal instability.

From the results of this study, it is concluded that wastewater from rice noodle manufacture could be used as a substrate for α-amylase production by *Aspergillus* sp. NU4. The enzyme was capable of hydrolysing raw starches not only of cereals but also of tubers and roots into dextrins, which can then be used directly in the production of ethanol, fructose, etc. It has a potential suitable for application in such starch processing industry. It also contributes to safe and economic waste management in the environment, where these waste continuously cause serious pollution problems.

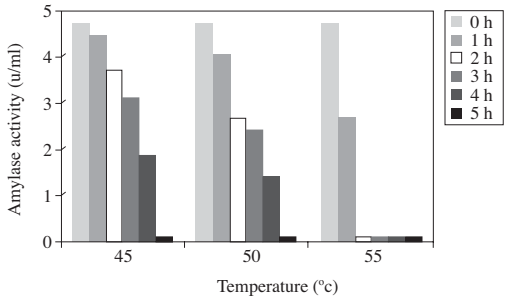

Figure 7. Thermal stabbility of α-amylase of *Aspergillus* sp. NU4.

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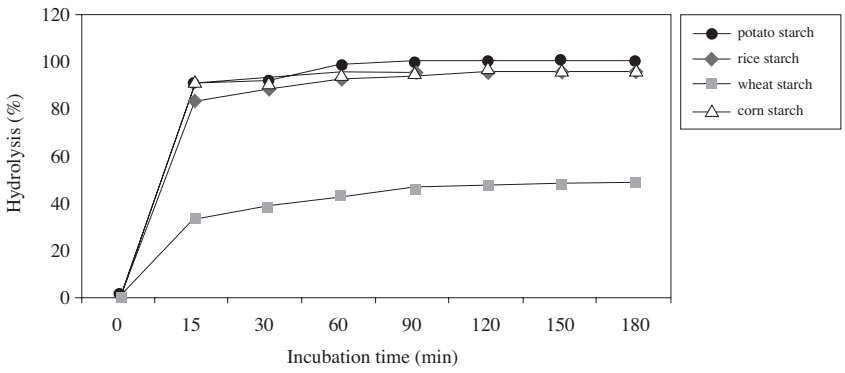

Figure 8. Hydrolysis of raw starch granules by crude α-amylase of *Aspergillus* sp. NU4.

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