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Amylatic Activity of Agaricus and Moulds for the Production of Bioethanol

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Abstract Fermentation of potato and carambola juice was investigated using Molds and Agaricus as a potential source of amylase. The amylatic activity of Moulds and Agaricus were studied and the effects of different parameters, such as starch concentrations, pH, incubation time, temperature were investigated. The maximum enzyme activity obtained for Moulds were 173 to 178U/g under the optimum conditions of an incubation period of 30min, 1.5 % starch solution, an incubation temperature of 60 ℃ and a pH of 5.0. For Agaricus, the highest amylase production was 14 to 16U/g using 1.5 % starch solution, pH of 6.0 incubated at 75 ℃ for 30 minutes. Reducing sugar was produced by fermentation of potato and carambola juice using mold and saccarification was conducted for production of bioethanol using yeasts. The ethanol produced from potato and carambola was approximately 11% (v/w) and 5% (v/v) respectively.

Keywords: *amylatic activity, mould, agaricus, bioethanol, fermentation*

1. Introduction

In the context of present energy crisis and environmental concerns, ethanol has constantly been an object of interest because of its potential as fuel. The largest single use of ethanol is as a motor fuel and fuel additive. Some Nations already required that gasoline be diluted with ethanol to help conserve fossil fuels [1]. When added with gasoline, ethanol reduces the volatile organic compound and hydrocarbon emissions, carcinogenic benzene and butadiene emissions, and particulate matter emissions from gasoline combustion. It is also used as beverage and starting material for a large number of chemicals. For this reason use of ethanol as fuel is increasing day by day.

Ethanol is produced from a wide range of feedstock at relatively low-cost. It can be synthesized from the petrochemicals, as well as by fermentation process. Due to the fast depletion of fossil fuel, the fermentation process is gaining more attention for the production of ethanol.

Ethanol fermented from renewable sources for fuel or fuel additives are known as bioethanol. Additionally, the ethanol from biomass-based waste materials is also considered as bioethanol. In conventional fermentation processes bioethanol is produced by fermenting grapes, sugar molasses and saccharifying rice, maize, sorghum, malts, potato etc. using the commercial standard amylases [2].

Amylases are the most important enzymes in present-day biotechnology. Although amylases can be derived from several sources, including plants, animals and microorganisms, microbial enzymes generally meet industrial demands. Currently, a large number of amylases are available commercially and they have almost completely replaced the chemical hydrolysis of starch in the starch processing industry [3]. The natural sources, such as, moulds and agaricus possess amylatic activity to certain extent. Their amylase activity should be evaluated to use them as a source of enzyme in fermentation process because they are easily available and they are inexpensive enzyme source.

Amylases can be produced either by submerged fermentation (SmF) [4,5,6] or solid-state fermentation (SSF) [7]. SSF holds tremendous potential for the production of enzymes [8]. It can be of particular relevance in those processes where a crude fermented product may be used as an enzyme source [9]. The selection of a particular strain, however, remains a tedious task, particularly when commercially significant enzyme yields are required. The use of SSF for the production of enzymes and other products has many advantages over SmF [10] and these have been widely discussed in the literature [8,10].

The applicability of the enzymes and their sources depend on some incubation conditions. The amount of amylase production by microorganism is dependent on the factors, such as incubation period, temperature, pH and also the medium substrate concentration.

Potato, a tuber, is a good source of carbohydrates, containing starch, cellulose, hemicellulose and pectic substances which are the main substrates for fermentation [2]. As a result waste potato is used worldwide for the production of ethanol. On the other hand Carambola (*Averhoa Carambola)*, a sour fruit, is a great source of vitamin C with a high amount of moisture content. The starch and sugar content of carambola were 1.41±0.34%

and 4.4±0.71% of the total edible portion respectively [11]. Sugars are easily fermented by enzymic catalytic mechanism using yeasts. The fruits are easily bio-degraded in normal atmospheric condition. So carambola should be a potential source for the production of ethanol.

In the present study the amylase activity of both Moulds and Agaricus strain were evaluated at different incubation conditions (starch concentration, pH, incubation time and incubation temperature) to determine the optimum incubation conditions for amylase production. Finally bioethanol was produced from potato and carambola juice using mould as a source of enzyme at the optimum incubation conditions. The amount of ethanol produced from potato and carambola juice was determined.

2. Materials and Methods

2.1. Materials

Sodium sulphite and Maltose ($C_{12}H_{22}O_{11}$) were purchased from LOBA Cheme., India. DNS (Dinitro Salicylic Acid), crystalline phenol, sodium potassium tartrate and propanone (acetone) were purchased from Merck, Germany. All working solutions were prepared by diluting the stock solution with distilled water. The Moulds (Black or Green) were isolated from decompost of breads available in the local market in Bangladesh. Agaricus was collected from the land where the soil is moist but no water gathered on the soil. The collected Moulds and Agaricus were preserved at 4℃ in a refrigerator. Yeasts (*Saccharomyces Cerevisiae*) were collected from the local market. The pH adjustments were made with HCl (Aldrich, Germany) or NaOH (Aldrich, Germany) solutions.

2.2. Study of Amylase Activity of Moulds and Agaricus Strain

2.2.1. Crude Enzyme Extract Preparation

The emzymic sample (Mould) was crushed thoroughly in a mortar with a pestle at ice-conditioned medium and weighed. The crushed sample was homogenized well with buffer solution of pH 5.0 (1g mould in 100mL solution). The mixture was stirred using a magnetic stirrer (BIDY STERLIN Co., England) for 10 minutes, filtered with few layers of cheese cloth and further clarified by centrifugation (FADA Medical Apparatus Factory, China) at 800rpm for 15 minutes. The clear supernatant was collected and used as crude enzyme extract. Similar method was followed for the extraction of Agaricus strain, except the pH of the buffer solution was 6.0 and homogenization was performed as for mould.

2.2.2. Enzyme Assay

To determine the amylase activity, the enzyme was assayed as taking 3 mL of crude enzyme extract in a conical flask with 6 mL of 0.1 M sodium acetate-acetic acid buffer (pH 5.0) and 3ml of 1wt% aqueous soluble starch solution for the Moulds and pH 6.0 buffer solutions was selected for Agaricus strain.

2.2.3. Method of Incubation

The enzyme assay of Moulds was incubated at 60℃ and that of Agaricus strain at 75℃ for 30 minutes. 3mL of aliquot of the extract was pipetted into a test tube and 3mL of DNS reagent was added to it. The mixture was heated in boiling water bath for 5 minutes. After the colour had developed, 1mL of 40% sodium potassium tartrate solution was added to the warmed tubes. A blank tube was also prepared by adding distilled water in place of crude enzyme extract following the same configurations. The amount of maltose was calculated by Uv-vis spectrophotometer (Shimadzu, Model UV-1650PC).

2.2.4. Measurement of Amylase Activity

Amylase activity was assayed using 1% of soluble starch solution as substrate [12]. The amylase activity was measured by estimating the amount of maltose released by it. One unit of amylase activity; was defined as the amount of enzyme required to release 1μg of maltose from starch per minute at the specified conditions.

The effect of starch concentration on the production of amylase was studied by carrying out the incubation at different starch concentrations at 60℃ for Moulds and at 75℃ for Agaricus strain for 30 minutes reaction period. The influence of initial pH on the production of enzyme was investigated by carrying out the incubations at different pH for 30 minutes period. The temperature was 60 ℃ for Moulds and 75 ℃ for Agaricus strain. Change in amylase activity with incubation time was also studied at pH 5.0 for moulds and at pH 6.0 for agaricus strain. The temperature was 60℃ and 75℃ respectively. Effect of incubation temperature on enzyme activity was studied at pH 5.0 for Moulds and at pH 6.0 for Agaricus strain with duration of 30 minutes incubation.

2.3. Production of Bio-ethanol from Potato and Carambola by Fermentation Method

2.3.1. Procedure for the Fermentation of Potato

Potatoes were collected for the fermentation using Moulds (grown on breads) as catalytic enzyme source. Potatoes were sliced in small pieces and then mashed. Required amount of distilled water was added with the mash and boiled in a water bath for an hour. 50g of boiled mash was diluted by adding water. A portion of Mould extract was added and the medium was cooled to 80-90℃ with constant stirring for one hour in order to liquefaction. The mash was allowed to cool to 60℃ for half hour. pH of the medium was adjusted to 5.0 ± 0.1 with the addition of phosphoric acid. The rest of the Mould extract was then added and the mash was kept at 60℃ for 90 minutes where saccharification of starch occurred. The mixture was then cooled to 30℃ and yeasts (*Saccharoces Cerevisae*) were put on it with constant stirring until the fermentation ends. To remove the exhaust of the produced carbon dioxide gas the gas outlet glass tube was sinked to the water in a beaker containing calcium oxide (CaO) solution. After the completion of fermentation the fermented product was filtered and then the filtrate was distilled at 94℃. The distilled product was again redistilled at 80℃. The amount of the distilled ethanol was measured by measuring the refractive index using a Refractometer (Abbe Refractometer).

2.3.2. Fermentation of Carambola Juice

The fermentation of carambola juice was carried out by the catalysation of enzymes from Moulds and Yeasts (*Saccharoces Cerevisae*). Moulds were used for saccharification of starch and yeasts were used to ferment the sugar to produce ethanol. The carambola fruits were cut into small pieces, blended by a blender and strained through a piece of white nylon cloth to extract the juice. The presence of sugar in the juice was characterized qualitatively by Fehling solution reduction test. The amount of reducing sugar was measured by Dinitrosalicylic Acid (DNS) method [13]. For the fermentation, 1000mL of juice was mixed with the required amount of distilled water in a 2000mL round bottom flask. The initial pH of the medium was maintained at 4.6 - 5.0. Sufficient amount of moulds were added for the complete saccharification of the starch. A nutrients solution for yeast growth was given as per required amount: (g/l) KH_2PO_4 5; $MgSO_4.7H_2O$ 1; NH_4Cl 1.5; KCl 1.7. The required amount of yeasts was given into the fermentation medium. The medium was stirred continuously using a magnetic stirrer [Model: BIDY STERLIN Co., England] until the fermentation ends. After the completion of fermentation the fermented product was filtered and distilled. The ethanol concentration was determined by a Refractometer (Abbe Refractometer).

3. Results and Discussion

3.1. Effect of Different Incubation Conditions on Enzyme Production

Different incubation parameters such as substrate concentration, initial pH of the medium, incubation time and temperature variation had profound effects on the enzyme production from Moulds and Agaricus strain. The plots representing these effects are shown from Figure 1 to Figure 4.

3.1.1 Effect of Substrate Concentration

Figure 1 represents the amylase activity of Moulds and Agaricus strain at various starch concentration.

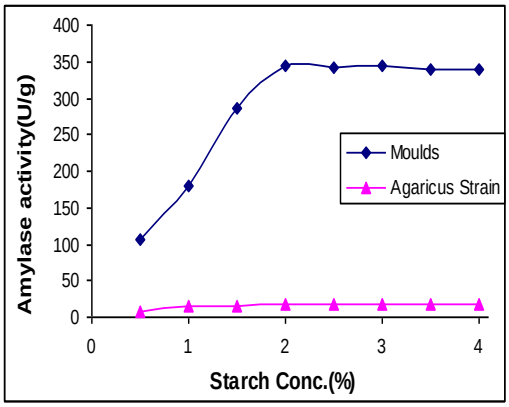

Figure 1. Amylase activity of Moulds and Agaricus strain at different starch concentration

It was seen from Figure 1, that the amylase activity for both mould and agaricus strain increases with starch concentration (%) to a certain extent and then starts to decrease. For moulds the amylase activity is optimum when the starch concentration is 1.5 - 2%. For agaricus strain the amaylase activity is optimum for starch concentration of 2 - 3%. The optimum amylase activity of mould was 314 ± 30U/g and for agaricus strain it was 17.57U/g.

3.1.2. Effect of pH

The effect of pH on the production of amylase from mould and agaricus strain is shown in Figure 2. The pH range was 3 - 10. Here also the amylase activity increases with pH to a certain extent. After that the amylase activity decreased.

From Figure 2 it was observed that, the Moulds shows greater activity at pH 5.0 having amylase production of 173.25U/g whereas the agaricus strain have the maximum amylase activity at pH 6.0 having amylase production of 15.0U/g.

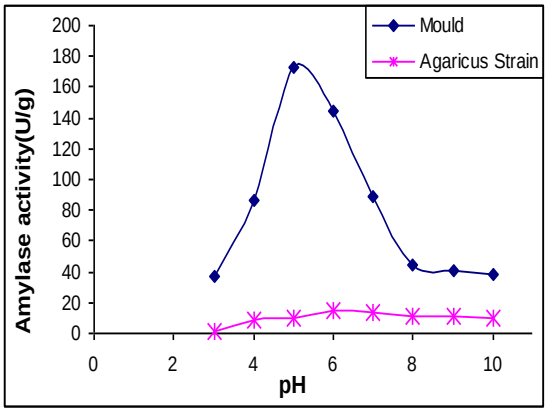

Figure 2. Effect of pH in amylase activity

3.1.3. Effect of Incubation Time

Incubation time has a significant effect on the production of enzyme. The effect of incubation time on the enzyme activity was studied at 60℃ and pH 5.0 for moulds and at 75℃ and pH 6.0 for agaricus strain. The observed results are represented in Figure 3.

From the observation of effect of incubation time it was found that the Moulds shows highest enzyme production of 171.75U/g after 30 minutes of incubation and agaricus strain shows the highest enzyme production of 14.84U/g after 30 minutes of incubation.

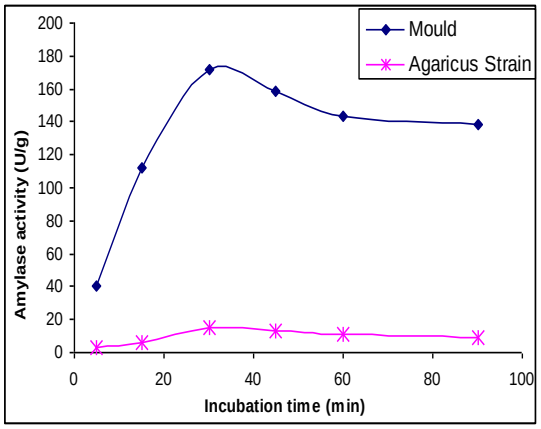

Figure 3. Effect of incubation time on amylase activity

3.1.4 Effect of Incubation Temperature

Effect of incubation temperature on the amylase activity was studied at optimum starch concentration, pH and incubation period for both moulds and agaricus strain. The results are presented in Figure 4. The maximum amount of enzyme (173.25U/g) is produced by Moulds at 60 ℃ of incubation temperature and agaricus strain shows the highest enzyme production (15.45U/g) at 75 ℃ of incubation temperature.

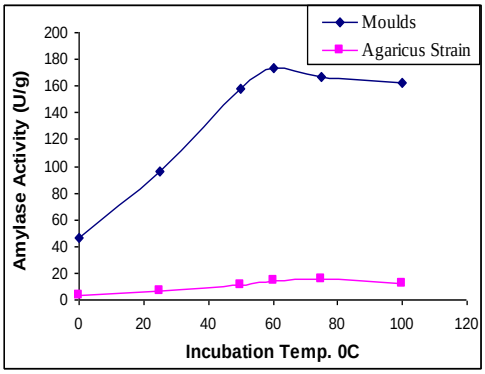

Figure 4. Effect of incubation temperature on the amylase activity

The optimum conditions for enzyme production from both moulds and agaricus strains are obtained from above observations and are listed in Table 1.

Table 1. optimum conditions for Enzyme production

	% of Starch	pH	Incubation Period(min)	Incubation Temp. (℃)
Moulds	1 - 2%	5.0	30	60
Agaricus Strain	2 - 3%	6.0	30	75

This study also indicates that the amylase activity is much higher for moulds than that of for agaricus strain. That is moulds are better source for enzyme production.

3.2 Ethanol Production by Fermentation

3.2.1 Fermentation of Potato and Carambola Juice

The start of fermentation process was confirmed by the production of CO_2. Fermentation was carried out for several days. Samples were taken out at different intervals. The pH of the fermentation medium of potato and carambola juice was measured after every 24 hours interval. The result of pH change with time is presented in Figure 5 which implies that due to the formation of lactic acid [2], the pH of the medium was continuously reduced with the time interval.

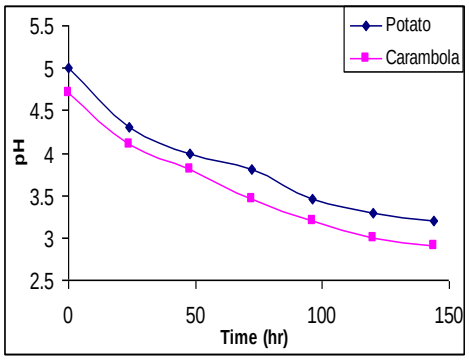

Figure 5. Change in pH with time during fermentation of potato and carambola juice

After the fermentation was completed the ethanol production from both potato and carambola were measured. It was found that approximately 11% (w/v) ethanol can be produced from potato by saccharification of potato starch using moulds on a 100 g weight basis. An overall of 5.0% (w/v) (approximately) ethanol was produced from the fermentation of carambola juice on a 100g weight basis.

4. Conclusion

The present study indicates that moulds and agaricus can be used as source of amylase for ethanol production. The amylase activity of moulds and agaricus strain increases with the increase in concentration of starch, pH, incubation period and incubation temperature to a certain extent and then starts to decrease. The maximum amylase activity of mould was found at 1-2% starch concentration, pH 5, 30 minutes of incubation time and 60 ℃ of incubation temperature and that of Agaricus strain was found at 2-3% starch concentration, pH 6, 30 minutes of incubation time and 75 ℃ of incubation temperature. This study also indicates that moulds are better source of enzyme then agaricus. Bio-ethanol was produced from potato and carambola juice by fermentation process. Mould was used as the source of enzyme. The overall amount of ethanol produced from potato and carambola juice was found to be 11% (w/v) and 5.0 %(v/v) respectively. Change in pH during fermentation was studied and it was found that the pH decreases with time due to the formation of lactic acid in the fermentation both.

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