RESEARCH NOTE

PRODUCTION OF GLUCOAMYLASE BY *ASPERGILLUS NIGER* UNDER SOLID STATE FERMENTATION

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Abstract In this study, Glucoamylase production by *Aspergillus niger* was investigated under solid state fermentation using low cost by-products of agricultural processes as substrate. Highest enzyme production was observed when a combination of wheat bran (WB) and corn flour (CF) was used as compared to WB+ rice bran, WB+ rice flour and WB alone. Several compositions of corn flours were experimented. The best combination of WB in addition of 10% corn flour resulted maximum enzyme activities. Study of the fermentations in flasks revealed 373.3 IU/gds and 41.4 g/l for the enzyme activity and protein concentration respectively under conditions of 30 $\pm$ 1^{o}C, pH 4.7 and time 96 h. Optimum conditions provided by flask experiments were applied to produce the enzyme in a fermenter. A tray type sterilized fermenter was designed (7-lit volume, 2 trays of 450x230x35 mm, and 2 l/min aeration). The fermentations were performed under aseptic conditions and resulted in enzyme activity and protein concentration of 400 IU/gds, 33.3 mg/gds respectively (under 30 $\pm$ 2^{o}C, pH=4.5 ~ 4.7, aeration 2 l/min, 90% relative humidity and time 72 h). The adequate amounts of enzyme activity, protein concentration and CO_2 evolution revealed the efficiency of the bioreactor in comparison with flasks.

Keywords Solid state fermentation; *Aspergillus niger*; Glucoamylase; Enzyme activity.

چکیده در این مقاله تولید گلوکوآمیلاز توسط آسپرژیلوس نایجر در شرایط کشت جامد به وسیله تخمیر حالت جامد محصولات فرعی فرایند های کشاورزی بررسی شده است. بیشترین تولید آنزیم با استفاده از پوسته گندم و آرد ذرت (۱۰٪) در مقایسه با بکارگیری پوسته گندم به تنهایی و یا همراه با آرد برنج و پوسته برنج مشاهده شد. بررسی تخمیر کشت جامد در فلاسک مقادیر ۳۷۳/۳ IU/gds فعالیت آنزیمی و ٤١/٤g/l غلظت پروتیئن را در شرایط ۳۰±۱ °C، pH اولیه حدود ٤/۷ و زمان ۹٦ h نشان می‌دهد. شرایط بهینه بدست آمده در مقیاس فلاسک در یک فرمانتور کشت سطحی نیز بررسی شد. فرمانتور سینی داری با قابلیت استریل شدن با ظرفیت ۲ سینی به ابعاد۳۵mm×۲۳۰×٤٥۰، حجم ۷ lit، گنجایش ۲۰۰ g سوپسترا و هوا دهی ۲ lit/min طراحی و به کار برده شد. تخمیر انجام شد و فعالیت آنزیمی ٤۰۰IU/gds و غلظت پروتئین ۳۳/۳ mg/gds در شرایط ۳۰±۲°C، pH ٤/۷–٤/٥، هوا دهی ۲ lit/min، رطوبت نسبی هوا ۹۰٪ وزمان ۷۲ h بدست آمد.در فرمانتور ساخته شده تلقیح اسپورها به محیط کشت در شرایط کاملا استریل و بدون باز شدن درب آن انجام گرفت. با توجه به نتایج این پژوهش، مقادیر مناسب فعالیت آنزیمی، میزان پروتئین تولیدی و CO_2 حاصل حاکی از کاربرد موثرتر فرمانتور در مقایسه با روش فلاسک بود.

1. INTRODUCTION

Glucoamylase (1, 4-α-D-glucan glucohydrolase) is one of the most commercially important starch enzymes. This enzyme is produced naturally by means of many molds and yeasts. Among the fungi group, *Aspergillus niger* has been the most commonly used one for the enzyme production. Production of glucoamylase (GA) is performed mostly by two methods, Solid State or Solid-Substrate Fermentation (SSF) and Submerged Fermentation (SMF) [1]. SSF processes are selected when the substrate is solid or starchy. That is, SSF is generally defined as the growth of microorganisms on solid materials [2]. No water solution is present but water is also associated with the solid components. However, the substrate must contain enough moisture, which is provided by evaporation and metabolic activity [3]. This method has been used since 3000 years BC in China. As a case in point, Miso is a fermented food in Japan, China, Taiwan, Philippines, and Indonesia. As another illustration, the soy sauce Koji has traditionally been prepared by SSF technique since ancient time [4]. The developments in SSF have been reviewed and advantages and disadvantages have been discussed in the literature [5 - 7]. From economic points of view, some advantages of SSF over SMF, can be described as: **a**- No waste production in the case of enzyme Koji fermentation, **b**- Less energy consumption in fungal $\alpha-$ amylase production, **c**- The longer production phase in amyloglucosidase production, **d**- The absence of coproduced carbohydrates, **e**- The shorter fermentation period in catalyses production, **f**- The use of waste or spent wheat bran in cellulase and xylanase production, **g**- The extra-cellular nature of the enzyme in $\alpha-$galactosidase production, **h**- No foam generation, **i**- Eliminating the need of solubilization of nutrient with substrate, **j**- Direct incorporation of plant material in solid media, **k**- The use of impure substrates in citric acid fermentation, and **l**- Elimination of the need for rigorous control of many parameters during fermentation [8].

Mathematical models have also been developed to overcome the problems associated with the SSF, and good agreement of the models with the experimental data were obtained [9, 10]. In another work, moisture content was accurately controlled by a designed sensor. This sterilizable sensor was useful to regulate the moisture content during fermentations and also has a great use in biocatalytical systems with much lower range of moisture content [11].

SSF was also used in small – scale for delignification of aspen wood with *phlebia tremellosa* and conditions were optimized [12]. Having the SSF systems, the researchers found that there were not enough opportunities for dynamic control in SSF. In other words, conditions must be arranged before the start to guide the fermentation along the desired path, and any deviations from the path are difficult to correct, while the fermentation is underway [13].

Selection of a suitable microorganism is one of the most important criteria in SSF. Several groups of microorganism such as filamentous fungi group can grow on solid substrates. Laboratory studies involving SSF have used flasks, beakers as bioreactors, while in large scale SSF works either tray type or drum type bioreactors have been mostly employed. Moreover, others have used column bioreactors. As an example, columns of two sizes were developed for GA production using *Aspergillus niger* (*A. niger*). Results showed that, the enzyme yielded in these bioreactors were higher than those obtained in flasks [14]. Studies show that agro-industrial residues are generally selected as an appropriate choice of medium in enzyme production by SSF [15]. Optimization of process parameters for amylase production using wheat bran has been already studied in SMF and SSF [16]. Moreover, utilizing combination of wheat bran, rice bran, and other rice components has been reported [17]. The aim of the present study was comparison of four natural substrates in the GA production in flasks and in a fermenter to reach the optimum conditions as well as comparison of two types of Iranian culture type of *A. niger*.

2. MATERIALS AND METHODS

2.1. SSF Bioreactor A modified tray type fermenter was used because of the simplicity and the large application in small-scale, semi commercial and commercial plants. Two

aluminium trays were arranged one above the other with a 10 cm gap between them. The air circulation was carried out by an air pump, from the bottom of the fermentation chamber, under the trays to the top. Before entering the chamber, the air inlet was passed through the filtration unit first, and then through the hot water chamber, which provided the appropriate conditions of humidity and temperature. The circulated air was measured by a rotameter. The volume of this fermenter was 7 liters with the capacity of 2 trays and 200 g substrate. The sizes of the trays were 450 x 230 x 35 mm. The substrate was entered to the sterilizable fermenter by a specific unit, which provided the distribution of the substrate in the trays. Then the sterilization took place (steam temperature of 121 ^{o}C, pressure of 1.5 bar and time of 30~45 min). After it was cooled down to 30 ^{o}C, the inoculation was performed by adding the inoculums to the fermenter, using the inoculants addition unit (Figure 1, No. 5). Moreover, temperature and humidity probes have been installed near the air outlet of the chamber. Finally, the fermenter was equipped with a heat jacket, which circulated hot or cold water when it is necessary to control the temperature (Figure 1).

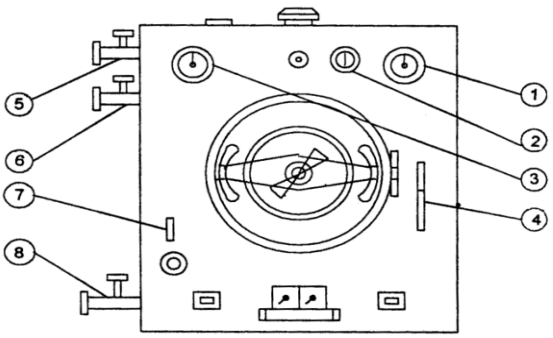

Figure 1. Schematic diagram of the fermenter
1- Pressure Control A, 2- On-Off key, 3-Pressure Control B, 4-Water Manometer, 5- Inoculants Addition Unit, 6-Air Exit Valve, 7-Condenser, 8-Air Inlet Valve

2.2. Microorganisms Two culture types of *A. niger* PTCC 5011 and PTCC 5012 were obtained from the microbial culture collection of IROST (Iran Research Organization of Science and Technology). Stock cultures were maintained on PDA (potato-dextrose-agar) medium. The cultures were preserved at 4^{o}C and renewed once a month. Then, the experiments were performed in flasks and the activity of the two *A. nigers* were tested under the same conditions (WB+ rice flour (RF)+ mineral solution, 18g, 2g and 1ml respectively as substrate, pH = 4.7, T = 30^{o}C, t = 96 h). The mineral solution composition was 3.357, 3.775, 0.424 and 114.65 mg/ml of Fe, Zn, Cu and N, respectively. Based on their activity, No. 5012 was chosen due to its higher activity comparing with No. 5011.

2.3. Solid Substrate In this study, commercial quality of natural substrates such as wheat bran (WB), rice bran (RB), rice flour (RF), and corn flour (CF), obtained from the local market, were tested. The size of the wastes was as follows: WB and RB were more than 1000 microns, and RF and CF were less than 180 microns in diameter. All the materials and substrates were sterilized under the appropriate conditions. Potato Dextrose Agar (PDA) was used as stock culture. The combination of substrate used for the flask experiments was 18g WB+ 2g CF and the mineral solution containing the aforementioned concentrations of Fe, Zn, Cu and N. In addition, the substrate for the fermenter was consisting of WB 90 g, CF 10g and the mineral solution (16.785, 18.875, 2.135 and 598.25 mg/ml of Fe, Zn, Cu and N, respectively). The reason for the choice of the substrate and the related experiments will be discussed later in this paper.

2.4. Enzyme Extraction A weighed quantity of the enzyme containing substrate (10 g) was treated with 50 ml distilled water and mixed in a shaker for 30 min. The whole contents were vacuum filtered, using Millipore filters (0.45 microns) and Buchner flask. The solids remained in the flask were again mixed with 50 ml distilled water and were shaken for 30 min in a similar way as above and filtered to get 50 ml filtrate. Both filtrates were combined and stored for the enzyme analysis. The obtained enzyme solution can be filtered three times if necessary. The color of the solution was brown.

2.5. Analytical Methods Reducing sugars produced were determined as glucose by dinitrosalicyclic acid method [18]. Enzyme activity unit (IU) is expressed as micromoles of reducing sugars (glucose) produced per minute by the total

amount of GA extracted from 1 g (dry weight) of substrate under the normal conditions. Direct determination of biomass is not possible under these conditions because of the penetration of fungal mycelia into solid substrate. Thus, the method of determination of cell glucoseamine was used. It was assumed that there is 50 mg glucoseamine for each gram of the cell, and the amount of glucoseamine as the cell mass was analyzed by the colorimetric method of Elson and Morgan at 540 nm wavelength [19 - 21].

2.6. Experiments in Flasks All the fermentations were carried out under the sterile hood near the flame to prevent contamination. Cultures were incubated for 4 days at 28-30^{o}C, then preserved at 4^{o}C. In this work, the culture was added to the substrate directly in the flask and 2 ml distilled water was added under sterile conditions. Then, it was incubated at 30 ± 1^{o}C for 96 h. Then, 10 g of the sample was washed with 50 ml distilled water, shaken and filtered. Afterwards, the pH of this solution was determined. As the control of pH in solid substrate is very difficult, a few experiments with different initial pH were made and the pH which yielded the best activity was selected (pH = 4.5-4.7).

2.7. Experiments in the Fermenter For the fermentation in reactor, 50 ml sterile distilled water was added to the culture and then mixed in the shaker. Spore concentration was 0.00035 pores/ ml. The initial pH was adjusted at 4.5-4.7 with 1 N HCI. After these preparations, the substrate was distributed on the trays and sterilization was performed using steam (T = 121^{o}C, P = 1.5 bar) for 30-45 min. When it was cooled to 30^{o}C and reached the relative humidity of %90, the culture was injected to the fermenter. Fermentation was started by passing the air through the fermenter and aeration rates of 1 lit/min and 2 lit/min were tested while other conditions were identical (substrate 100g, salts 5 ml, acid 1.2 ml). It was revealed that the aeration of 2 lit/min gave better results.
Fermentation kinetics is sensitive to the variation in ambient and internal gas composition. Therefore, the amount of CO_2 gas was analyzed during the enzyme production to investigate the reaction sensitivity to CO_2 gas. Carbon dioxide of the air outlet of the fermenter was trapped in 2 M NaOH solution. It was determined by carbonate precipitation with $BaCl_2$ and titration of the residual NaOH with 1N HCl, using thymol blue as indicator [12].

3. RESULTS AND DISCUSSION

3.1 The Effect of Substrate Literature reports that some researchers such as Pandey [22] used different substrates which confirmed that WB produced higher enzyme productivity and the partial replacement of WB with other substrates showed varying trend of enzyme production. Also, it has been stated that maximum enzyme activities were obtained when WB is mixed with corn flour. The same result was gained at the present study which is shown in Figure 2. WB+CF with the activity of about 350 IU/gds (gds sands for gram dried solid), was shown to be the most suitable substrate in this work. It is shown that increasing the amount of CF up to 15% would increase the activity up to a limit, which is shown in Figure 3. Comparison of the results confirmed that, the best activity was obtained for WB + 10 %CF (e.g. 18 g WB + 2 g CF).

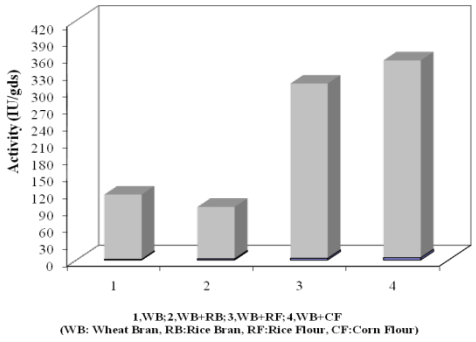

Figure 2. Effect of various substrates on enzyme activity

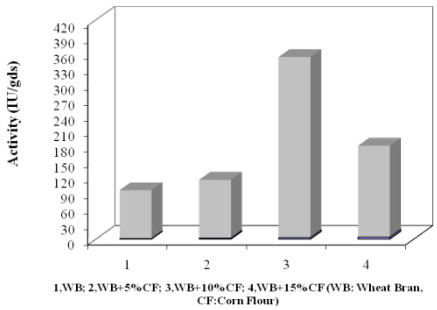

Figure 3. Effect of the percentage of CF on the enzyme activity

3.2. The Effect of pH As it has been stated before, production of Glucoamylase from *A. niger* seems to be sensitive to pH. This study revealed that SSF work in the fermenter requires the adjustment of pH at the start of the fermentation as some other researchers stated [13]. This adjustment was performed by adding selected amount of 1 N HCl (1.2 ml) to the substrate before entering the fermenter. Under such conditions, an increase in the activity and a decrease in the pH variations were appeared. In the present study, the initial pH range of 4.5 – 4.7 was considered to be the best pH for the growth of microorganisms in flasks. As Figure 4 showed, first, the pH decreased to 4.5 due to the production of organic acids, and then as the substrate and nutrients were used up, the consumption of organic acids caused an increase in pH.

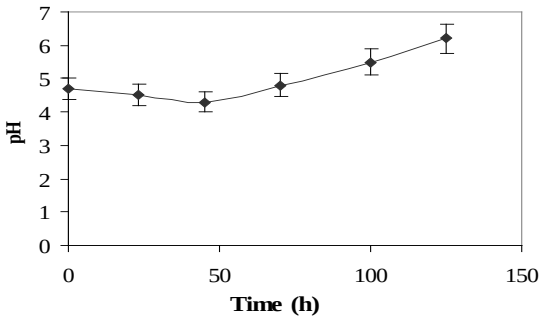

Figure 4. Variation of pH with time during the fermentation in flask

3.3. Results of the Experiments in Flasks The GA enzyme production with time was studied in flasks (Figure 5). As it is shown, the enzyme activity (IU/gds) increased up to 96 h of growth then decreased sharply. Maximum enzyme activity was 373.3 IU/gds under optimum conditions of 30±1 ºC, pH 4.7 and time 96h. The same variations were observed when the protein concentration was detected during time according to Figure 6. The protein concentration increased with respect to time up to 72 h then decreased. The maximum amount of protein was 41.4 mg/l under 30±1 ºC, pH 4.7 and time 72h. (Figure 6)

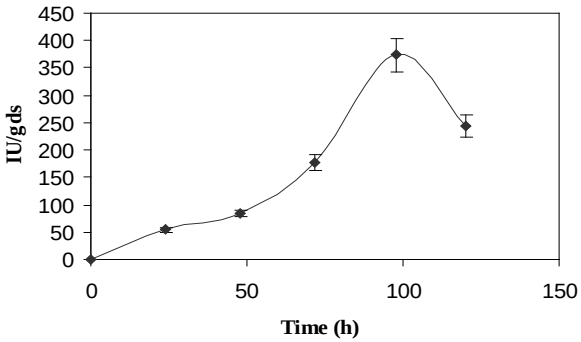

Figure 5. Variation of activity during the fermentation in flask

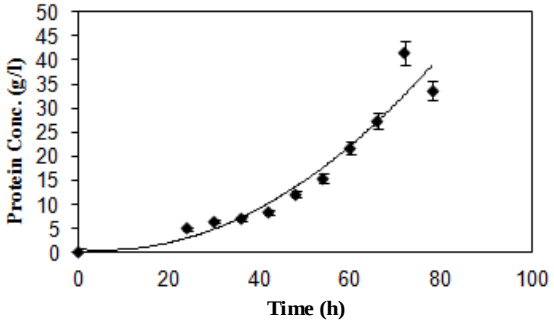

Figure 6. Variation of protein concentration during fermentation in flask

3.4. Production of Glucoamylase in a Tray Fermenter The designed tray fermenter (7 lit volumes, 2 trays 450 x 230 x 35 mm, 2 lit/min aeration, 90% relative humidity) was used to run fermentation. Figures 7 and 8 represent the variations of protein content, the activity, pH and cell mass via time respectively in the fermenter. The protein concentration, the enzyme activity and the cell mass values revealed an upward trend of enzyme formation during the fermentation until 72 h and then decreased due to the loss of substrate and inactivity of the *A. niger*. Maximum values of the activity, protein and cell mass were 400 IU/gds, 33.3 mg/gds and 4.5 g/gds respectively. The pH variations showed a quite smooth range (4.7~5.9), it decreased to 4.3 until first 36 h, then increased up to 72 h. Figure 9 shows the variation of mmols of CO_2 per gram of substrate during the time of fermentation. Therefore, the increasing amount of CO_2 confirms that the reaction and the enzyme production are proceeding.

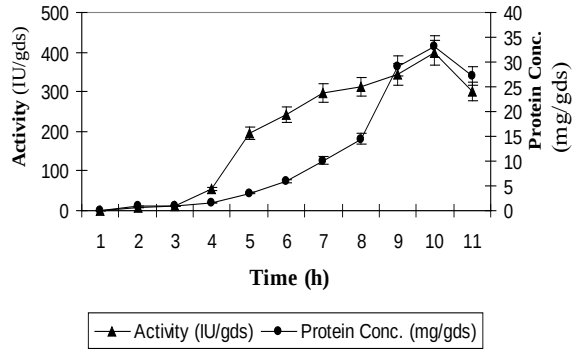

Figure 7. Variation of protein content and activity with time in the fermenter

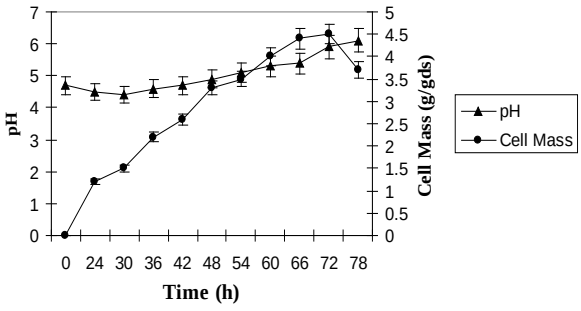

Figure 8. Variations of pH and cell mass with time in the fermenter

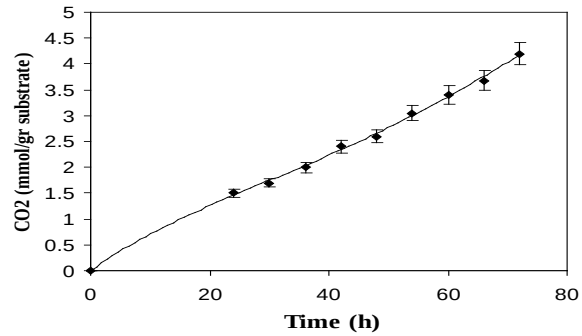

Figure 9. Carbon dioxide evolution during fermentation

4. CONCLUSION

In general, the results of the present study showed that adding CF (61% starch) as a strong carbon source to WB (14% starch) improves the enzyme activity rather than adding RF and RB. However, testing various percentages of CF showed that increasing high amounts of the carbon source had an inhibitory effect on enzyme activity. Thus, the optimum substrate was shown to be WB+10%CF. Experimental results confirmed that the designed fermenter was effective for producing GA from *A. niger*. They show that cell mass increases up to 72 h and then decreases because of finishing the substrate, and also inactivity and death phase of the *A. niger* fungi. This fermenter is also recommended for the production of acids such as citric acid. An important advantage of the fermenter employed in the present study was that all the inputs could be entered to the fermenter under completely sterile conditions, and there was no need to open the reactor. Studies on the optimizing the relative humidity of supply air and the aeration will be carried out in this laboratory as the further work.

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