Research Article

Beta-D-fructofuranosidase production by *Aspergillus niger* IBGE 01 using shaken flask technique of submerged fermentation

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Abstract

The modern biotechnological set up has motivated the need of enlarged survey of microorganisms which could be utilised in the extreme conditions of industries. In this work optimization parameters in submerged fermentation were studied for the production of Invertase from *Aspergillus niger* IBGE 01 using agricultural wastes (sunflower waste, cotton stalk and rice husk) as well as agro industrial wastes (date syrup and molasses) as sources of carbon. Effects of incubation time period (24-240 h), various cultural media (CM1, CM2, CM3, CM4 and CM5) sources of nitrogen (Corn steep, Casein, Potassium Nitrate Albumin Ammonium Sulphate Urea and Yeast Extract), pH (4.0-9.0), temperatures (30-70° C), inoculum size (4×10^6 - 8×10^6 conidia/mL) and agitation rate (50-300 rev/min) were also investigated for maximum invertase production. Optimal conditions for the production of invertase (8.23 U/mL) by *Aspergillus niger* IBGE 01 were observed when the strain was grown on culture medium CM1 containing yeast extract as a source of nitrogen, molasses as a source of carbon after 72 h of incubation at 40° C, initial pH 6.0, inoculum size of 5×10^6 conidia in 50 mL of culture medium and agitation rate of 150 rev/min. The strain was proved pH (up to 9) and thermo stable (up to 60° C) therefore can be used in industries for invertase production.

Key words: Invertase; *Aspergillus niger*; Optimization.

Introduction

The modern biotechnological setup due to increasing demand of enzymes has motivated the need for enlarged survey of microorganisms surviving and producing enzyme in extreme conditions [1]. For the production of large quantities of enzymes filamentous fungi have biotechnological

importance [2].

Invertase (Enzyme Code 3.2.1.26), splits sucrose into glucose and fructose. It is one of the most widely used enzymes by food industry in making chocolate covered cherries. This enzyme is also used in paper industry and to make artificial honey in which it contributes to anti-bacterial

properties [2, 3].

In the present work specific interest has been focused on agriculture waste like sunflower cotton stalk rice husk date syrup and molasses because they are usually related with pollution. Being the cost effective sources of carbon agricultural wastes have a potential for conversion into useful products [1]. In this work the secretion of invertase by *Aspergillus niger* IBGE 01 in submerged fermentation with all optimised parameters are being reported because no work is done to optimize all parameters for the production of invertase from this strain.

Materials and Methods

Aspergillus niger IBGE 01 was obtained from the Institute of Biotechnology & Genetic Engineering University of Sindh and the culture was maintained as followed by Dahot [4].

Optimization of parameters

All experiments were done in such a way that the parameter optimized in one experiment was fixed in the following experiments.

Culture medium

First of all the most suitable culture medium was determined. Composition of various culture media were (in g/L)

CM1: Dextrose 10, Peptone 5, Epsom salt 5, KH_2PO_4 5, Common salt 2.5 and ferrous sulphate hepta hydrate 0.01 [5].

CM2: Yeast extract 10, peptone 20 and sucrose 20 [6].

CM3: Yeast extract 20, peptone 40, sucrose 20, KH_2PO_4 2 and Epsom salt 1 [7].

CM4: NaNO_3 3, KCl 0.5, Epsom salt 0.5, ferrous sulphate hepta hydrate 0.01, K_2HPO_4 1, Sucrose 30 [8].

CM5: Sucrose 40, Corn steep liquor 30, NaNO_3 3, KH_2PO_4 0.5, Epsom salt 0.05, CaCO_3 2.5 [9].

Incubation time

After the determination of the most suitable culture medium, optimum incubation time period was determined. It was done by

growing the strain on CM1 at various interval of time from 24-240 hours.

Carbon sources

After the optimization of incubation time the most suitable carbon source was determined. It was done by replacing the glucose (control) of CM1 by various wastes including sunflower waste, cotton stalk, rice husk which were hydrolysed by 0.3 N H_2SO_4 and 0.6 N H_2SO_4 . Date syrup and molasses were used 0.5 % and 1 % in place of glucose (control).

Nitrogen sources

After the determination of the most suitable carbon source various nitrogen sources were checked for optimum production of invertase. It was done by replacing peptone of CM1 by Corn steep, Casein, Potassium Nitrate Albumin Ammonium Sulphate Urea and Yeast Extract.

Incubation temperature

The most suitable culture medium CM1 was tested on varying temperature from 30-70° C to determine the most suitable incubation temperature.

Initial pH of medium

The initial pH of a medium has an effect on growth and productivity of microorganism. A range of pH between 4.0-9.0 was checked for optimum production.

Inoculum size

Productivity was also checked in terms of No. of conidia/mL in 50 mL of optimised culture medium in order to obtain the optimized inoculum size of culture medium. The No. of conidia/mL was counted by haemocytometer.

Agitation rate

Effect of Agitation rate was also checked for optimization at 50, 100, 150, 200, 250 and 300 rev. /minute in orbital shaking incubator.

Determination of enzyme activity

Invertase activity was determined by BernFeld method [10]. One unit of invertase activity is the amount of enzyme which releases 1 μg of reducing sugar at 37° C per minute.

Results and Discussion

Effect of culture media

Effects of various culture media on invertase production by *A. niger* IBGE 01 after 24 h, at 30° C, initial pH 6.0, inoculum size 4×10^6 conidia and agitation rate 50 rev/min are poltted (Fig.1). The strain was grown on five different culture media *i.e.* CM1, CM2, CM3, CM4 and CM5. It was capable of growing well on all types of culture media but production of invertase was maximum (1.87 U/mL) on culture medium CM1 containing (g/L) dextrose 10, peptone 5, epsom salt 5, KH_2PO_4 5, common salt 2.5, ferrous sulphate hepta hydrate 0.01, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.002, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 0.001 and thiamine hydrochloride 0.001. CM1 was selected for the next study of invertase production.

Selection of the most suitable culture media has the deep effect on enzyme production. Many researchers all over the world have reported different culture media for maximum invertase production [6-9, 18].

Effect of incubation time period

Effects of incubation time periods on invertase production by *A. niger* IBGE 01 in CM1 at 30° C, initial pH 6.0, inoculum size 4×10^6 conidia and agitation rate 50 rev/min are poltted (Fig. 2). Invertase activity was measured at regular interval of 24 h and it was found that the maximum activity (2.34 U/mL) was observed after 72 h of incubation. On prolonged incubation enzyme activity was decreased, which may be due to denaturing of enzyme or synthesis of inhibiting metabolite [1]. Incubation time period of 72 h was reported for invertase production by *Alternaria tenuis* and *Saccharomyces crevisiae* [9] while 96 h by *Aspergillus ochraceus* [11].

Effect of carbon sources

Effects of various carbon sources on invertase production by *A. niger* IBGE 01 after 72 h in CM1 at temperature 30° C, initial pH 6.0, inoculum size 4×10^6 conidia

and agitation rate 50 rev/min are shown in Fig. 3. In the present work date syrup, molasses, sunflower waste, cotton stalk and rice husk have been used as sources of carbon, which were (except date syrup and molasses) hydrolyzed by 0.3N and 0.6N of H_2SO_4 . It was observed that invertase activities were lower than control (2.34 U/mL) in case of 0.3N sulphuric acid hydrolysed agriculture waste (0.85, 1.12 and 1.48 U/mL for cotton stalk, sunflower waste and rice husk respectively) and higher with 0.5 % of date syrup and molasses (2.58 and 3.06 U/mL respectively). Invertase activities were closer to control, glucose (2.34U/mL) when 0.6N sulphuric acid hydrolysed agriculture waste (1.86, 1.82 and 2.09 U/mL for cotton stalk, sunflower waste and rice husk respectively) and enzyme activities were higher when 1 % of date syrup (2.57 U/mL) and molasses (3.78 U/mL) were used. A number of nonconventional carbon sources such as starch, oilcakes, cassava starch, potato, corn and tapioca have been also used in submerged fermentation for various enzymes production [12-15, 20, 22].

Effect of nitrogen sources

Effects of various nitrogen sources on invertase production by *Aspergillus niger* IBGE 01 after 72 h in CM1 containing molasses as carbon source at 30° C, initial pH 6.0, inoculum size 4×10^6 conidia and agitation rate 50 rev/min are poltted (Fig. 4). Various nitrogen sources (casein, corn steep liquor, potassium nitrate, albumin, ammonium sulphate, urea and yeast extract) were used in the quantity of 0.25 and 0.50 % in place of peptone (control having enzyme activity 3.91 U/mL). The strain showed the capability of utilizing well all types (except urea) of nitrogen sources but yeast extract was found to be the best (3.81 U/mL in 0.25 % and 4.58 U/mL in 0.50 %). Increase in enzyme activity may be due to the reason that various other enzymes are also produced, which may affect at the

production of each other [16]. Yeast extract was also reported as the best nitrogen source for *Candida utilis*, *Saccharomyces cerevisiae* [6] and *Aspergillus ochraceus* [11]. Very low values (0.26 U/mL in 0.25 % and 0.09 U/mL in 0.5 %) of invertase activities were observed when urea was used as nitrogen source. It may be due to denaturing effect of urea on invertase [17].

Effect of temperature

The effects of incubation temperatures on invertase production by *Aspergillus niger* IBGE 01 after 72 h in CM1 containing molasses as carbon source, yeast extract nitrogen source, at initial pH 6.0, inoculum size 4×10^6 conidia and agitation rate 50 rev/min are presented (Fig. 5). The fermentation medium was incubated at a range of temperatures 20-70° C. Invertase activity was the highest (5.67 U/mL) at 40° C. Similar optimum temperature has been reported for *P. lilacinum* [2].

Effect of initial pH

The effects of initial pH of fermentation medium on invertase production by *A. niger* IBGE 01 after 72 h in CM1 containing molasses as carbon source, yeast extract nitrogen source, temperature 40° C, inoculum size 4×10^6 conidia and agitation rate 50 rev/min are shown (Fig. 6). A range of pH (4.0 to 9.0) was studied and found that initial pH of 6.0 would be the best for maximum enzyme production (5.67 U/mL). Similar initial pH 6.0 was also reported by Dworschack & Wickerham [6] for invertase production from *Saccharomyces cerevisiae*. Results also indicate that invertase production is appreciable between pH 5-8, which shows its pH stability that is a requirement for industrial use of a microorganism [1].

Effect of inoculum size

Effects of inoculum sizes on invertase production by *A. niger* IBGE 01 after 72 h in CM1 containing molasses as carbon source, yeast extract nitrogen source, temperature 40° C, at initial pH 6.0 and agitation rate 50 rev/min are presented (Fig. 7). The quantity of enzyme produced by a fungus depends upon number of conidia added to the culture medium. Flasks were added with 4×10^6 - 8×10^6 conidia and maximum invertase activity (7.01 U/mL) was observed when 5×10^6 conidia were added to the medium. Literature survey revealed that researchers have used inoculum sizes on the basis of percentage of fermentation media [1, 4, 11] but in the present study counted conidia were added to the medium in order to generate more accurate results. Large inoculum size causes overgrowth and nutritional imbalanced resulting less production of enzyme [1, 4, 11, 21].

Effect of agitation rate

The effects of agitation rates on invertase production by *A. niger* IBGE 01 after 72 h in CM1 containing molasses as carbon source, yeast extract nitrogen source, 40° C, at initial pH 6.0 and inoculum size 5×10^6 conidia are exhibited (Fig. 8). Agitation rate is another factor which affects the enzyme production. Agitation of media ensures the homogeneous supply of nutrients [27]. The fermentation medium was agitated at 50, 100, 150, 200, 250 and 300 rev/min. Invertase activity was maximum (8.23 U/mL) at 150 rev /min. Literature survey revealed that researchers reported various agitation rates (100-200 rev/min) for enzymes production by different microorganisms [4, 19, 23-26].

Fig.1. Effects of various culture media on invertase production by *Aspergillus niger* after 24 h, at 30° C, initial pH 6.0, inoculum size 4×10^6 conidia and agitation rate 50 rev/min.

Fig. 2. Effects of incubation time periods on invertase production by *A. niger* in CM1 at 30° C, initial pH 6.0, inoculum size 4×10^6 conidia and agitation rate 50 rev/min.

Fig. 3. Effects of various carbon sources on invertase production by *A. niger* after 72 h in CM1 at 30° C, initial pH 6.0, inoculum size 4×10^6 conidia and agitation rate 50 rev/min.

Fig. 4. Effects of incubation temperatures on invertase production by *A. niger* after 72 h in CM1 containing molasses as carbon source, yeast extract nitrogen source, at initial pH 6.0, inoculum size 4×10^6 conidia and agitation rate 50 rev/min.

Fig. 5. Effects of incubation temperatures on invertase production by *A. niger* after 72 h in CM1 containing molasses as carbon source, yeast extract nitrogen source, at initial pH 6.0, inoculum size 4×10^6 conidia and agitation rate 50 rev/min.

Fig. 6. Effects of initial pH of fermentation medium on invertase production by *A. niger* after 72 h in CM1 containing molasses as carbon source, yeast extract nitrogen source, temperature 40°C , inoculum size 4×10^6 conidia and agitation rate 50 rev/min.

Fig. 7. Effects of inoculum sizes on invertase production by *A. niger* after 72 h in CM1 containing molasses as carbon source, yeast extract nitrogen source, at 40°C , initial pH 6.0 and agitation rate 50 rev/min.

Fig. 1. Effects of agitation rates on invertase production by *A. niger* after 72 h in CM1 containing molasses as carbon source, yeast extract nitrogen source, at 40°C , initial pH 6.0 and inoculum size 5×10^6 conidia.

Conclusion

In the present study local fungus *Aspergillus niger* IBGE 01 was used and optimization parameters were determined for maximum invertase production by using various agricultural/industrial wastes as sources of carbon. Optimal conditions for the production of invertase (8.23 U/mL) by *Aspergillus niger* IBGE 01 were observed when the strain was grown on culture medium CM1 containing yeast extract as a source of nitrogen, molasses as a source of carbon after 72 h of incubation at 40° C, initial pH 6.0, inoculum size of 5x10⁶ conidia in 50 mL of culture medium and agitation rate of 150 rev/min. The strain was proved pH (up to 9) and thermo stable (up to 60° C) therefore can be used in industries for invertase production.

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