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Original Article

OPTIMIZATION OF CULTURE PARAMETERS FOR α-GLUCOSIDASE PRODUCTION FROM PROTONEMAL BIOMASS OF *BRYUM CORONATUM* SCHWAEGR

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ABSTRACT

Objective: The purpose of the present investigation was to optimization of culture parameters for α-glucosidase production from protonemal biomass of *Bryum coronatum* Schwaegr.

Methods: Fresh unopened, mature capsules were used as explant and the protonema that developed from the aseptic spores were cultured grown on 1/4th Murashige and Skoog basal medium. Frozen protonemal biomass was homogenized in 100 mM phosphate buffer (pH 7.0). The supernatant was assayed for α-glucosidase activity. Various culture parameters such as incubation period, temperature, pH, agitation, carbon sources and nitrogen sources were evaluated and further Taguchi orthogonal array method was performed.

Results: It was observed that both protonemal biomass and α-glucosidase production were maximum at 28 d of culture. Optimization of culture parameters such as pH, temperature and agitation speed for α-glucosidase production was found to be 6.0, 35 °C and 150 rpm, respectively. Among nutritional parameters, sucrose as carbon source and ammonium nitrate as nitrogen source found to be effective for enzyme production and maximum growth of protonemal biomass of *B. coronatum.* Based on Taguchi orthogonal array method, the optimal condition and their contribution on α-glucosidase production were evaluated as follows: sucrose (1.5%), ammonium nitrate (1%), pH (6.0), temperature (35 °C) and agitation (150 rpm).

Conclusion: In this study, the culture of moss, *B. coronatum* proved to be a good source for the enzyme α-glucosidase production. Temperature 35 °C and pH 6.0 were found to be optimum for maximum α-glucosidase production with respect to protonemal biomass production. Optimization of culture medium by Taguchi method has resulted in an increase in the α-glucosidase activity from 3.84-14.39 U/ml.

Keywords: *Bryum coronatum*, Protonemal biomass, α-glucosidase.

INTRODUCTION

α-Glucosidase (EC 3.2.1.20) release α-glucose from the non-reducing ends unit of substrates such as α-glucosides, oligosaccharides and starch [1]. This enzyme commonly associates with other amylolytic enzymes, which completely degrade and utilize starch as a carbon source [2]. It is ubiquitous in nature and plays an important role in *in vivo* processing of oligosaccharides of glycoproteins. α-Glucosidase is important n-glycosylation enzyme play an important role in n-glycosylation pathways and help in the proper folding of protein. This n-glycosylated enzyme is extensively distributed in eukaryotes and prokaryotes and exists in microorganisms as extracellular, intracellular, or cell bound enzymes, where they play important roles in uptake/utilization of nutrients and post-translational modification processes [3, 4]. Although this enzyme was isolated from various sources have been purified and their properties were studied, the nature of this enzyme is still not known in plants, especially in lower plant. On the other hand, it was reported that the n-glycosylation in the moss *Physcomitrella patens* is organized similarly to that in higher plant [5]. It is, therefore, of interest to investigate the production of this enzyme from moss, *B. coronatum*, which is dominantly found in BIT, Mesra campus, Ranchi, India.

B. coronatum Schwaegr. belongs to family Bryaceae (Bryopsida) which are densely tufted, yellowish-green plant. This moss is widespread in tropical to warm-temperate regions Asia, Africa, North America, South America, Australia, and Oceanic islands. It habitats on soil, bricks, cemented bricks and rocks. The aim of this paper is to report the optimum condition of culture parameters for α-glucosidase production with respect to growth of protonemal biomass of *B. coronatum*.

MATERIALS AND METHODS

Plant material and growth conditions

Mature capsules of *B. coronatum* were sterilized with 4% (w/v) sodium hypochlorite for 1 min, and washed three times with sterilized water. Spores within the capsules were aseptically removed and grown on medium with one-fourth concentration of MS (Murashige and Skoog) basal medium [6]. The protonema that developed from the aseptic spores were cultured on fresh medium at pH 5.8 and temperature 22 °C with 16/8h: light/dark condition. *In vitro* culture of *B. coronatum* was established from disinfected spores on MS medium of 1/4th strength with 0.7% (w/v) agar [7].

Extraction of enzyme from protonemal biomass of *B. coronatum*

Frozen protonema biomasses of *B. coronatum* were homogenized in 100 mM phosphate buffer, pH 7.0 containing 10 mM β-mercaptoethanol using a homogenizer for 15 min at 4°C. The homogenate sample was then, sonicated by using amplitude (35%) and energy 15,000 Joule with pulse rate 10 s on and 10 s off for 10 min at 4 °C and was centrifuge at 15,000 rpm for 15 min at 4°C. All subsequent steps were conducted at 4 °C and protein was extracted from the homogenate of protonemal cells [2]. The supernatant was used as a crude enzymatic extract. The supernatant was assayed for α-glucosidase and protein concentration. The protein concentration was determined against bovine serum albumin as the standard [8].

Assay of α-glucosidase enzyme activity

For α-glucosidase activities, the reaction mixture containing 10 mM p-nitrophenyl-α-D-glucopyranoside as substrates with 0.55 mL of 50 mM Na-acetate buffers (pH 4.5) was incubated at 40°C for 30 min. The reaction was stopped by the addition of 2 ml of 200 mM Na_2CO_3 and the colour developed by p-nitrophenol liberation was measured at 405 nm [9]. One unit of enzyme activity (U) was defined as the amount of enzyme releasing 1 µ mol of p-nitrophenol from the substrate per minute under standard assay condition.

Optimization of culture conditions for α-glucosidase production

Various process parameters that influence α-glucosidase production by protonema of *B. coronatum* were evaluated using the basal

1/4thMS medium. Enzyme production was carried out in 100 ml Erlenmeyer flasks containing 40 ml medium with 10 mg protonemal biomass.

Optimization of incubation period

For determination of protonemal biomass, 10 mg of protonema biomass of *B. coronatum* was used as inoculum and taken in 100 ml Erlenmeyer flasks containing 40 ml basal 1/4thMS medium (pH 5.8). Each culture was incubated at temperature (22 °C) on a rotary shaker set at 150 rpm for 42 d. The sample was harvested at 7-day intervals and production of protonemal biomasses was measured. Samples were withdrawn at 7-day interval and centrifuged at 15000 rpm for 15 min at 4 °C. The supernatants were assayed for α-glucosidase activities.

Optimization of the initial pH

To optimize the initial pH of the medium, the pH of the basal 1/4thMS medium was varied from 4.0 to 7.0 with 1N HCl or 1N NaOH. Incubation was carried out for 28 d at 22 °C temperature on a rotary shaker set at 150 rpm.

Optimization of incubation temperature

Optimal temperature for the maximum production of α-glucosidase was evaluated by incubation at temperature 18, 22, 26, 30, 35, 40 and 45 °C. Cultures were incubated for 28 d at the required temperatures in an incubator cum orbital shaker set at 150 rpm.

Optimization of agitation

Optimal agitation speed (rpm) for production of protonemal biomass and the maximum production of α-glucosidase were evaluated by using 70, 90, 110, 130, 150, 170 and 190 rpm agitation. Samples were withdrawn after 28 d of incubation at 22 °C.

Effect of various carbon sources

To determine the effect of various carbon sources such as dextrose, sucrose, lactose, xylose, maltose and galactose were added at 1% (w/v) level to the basal 1/4thMS medium. The incubation was carried out for 28 d at 22 °C on a rotary shaker set at 150 rpm.

Effect of various nitrogen sources

To determine the effect of various nitrogen sources such as such ammonium chloride, ammonium nitrate, potassium nitrate, ammonium phosphate and sodium nitrate were added at 0.5% level (w/v) to the basal 1/4th MS medium. The incubation was carried out at 22 °C for 28 d on a rotary shaker set at 150 rpm.

Statistical analysis

Protonemal biomass and α-glucosidase production values were presented as mean±standard deviation from five experimental data sets. Further, Taguchi orthogonal array method was performed. Five different factors and their concentrations at four different levels were selected for further evaluation for this enzyme (table 1).

The L-16 orthogonal experimental design along with α-glucosidase production values was presented in table 2. Qualitek-4 software (version 17.1.0, Nutek Inc., USA) for automatic design of experiments using the approach of Taguchi methodology was used in the present study.

Table 1: Factors and their levels employed in the Taguchi's experimental design for productions of α-glucosidase

Factors	Level 1 (L1)	Level 2 (L2)	Level 3 (L3)	Level 4 (L4)
Sucrose (%, w/v)	0.5	1	1.5	3
NH_4NO_3 (%, w/v)	0.5	1	1.5	2
Temperature (°C)	25	30	35	40
pH	5.5	6	6.5	7
Agitation (rpm)	110	130	150	170

RESULTS AND DISCUSSION

Determination of protonemal biomass at different time periods

Production of biomass depends on growth time period of culture in culture media which varied from species to species. It was found that maximum growth of protonemal biomass was at 28 d of culture, thereafter no further noticeable improvement of protonemal biomass with an increase in culture time period (Fig.1). It was found that for α-glucosidase, the highest enzyme activity present at 28 d in culture, after that enzyme activity decreased with increase in culture time period (fig. 1). It was also observed that both protonemal biomass and enzyme production were maximum at 28 d of culture, therefore, later on all samples were taken at 28 d of cultures.

Optimization of the initial pH of the culture medium

The pH of the culture medium influences the growth and subsequent metabolic product formation. Higher α-glucosidase production was observed at pH 6.0. With increase or decrease in an initial pH of the culture medium, it resulted in the decrease in activity of the produced enzyme. It also found that maximum protonemal biomass were at pH 6.0 of culture medium (fig. 2). In general, it was reported that bacterial α-glucosidase has neutral optimum pH of range 6.0 to 7.5 [10-12], whereas in few other cases such as *Bacillus* sp. KP 1035 and *B. licheniformis* KIBGE-IB4 optimum of pH was about 5.0 [13, 14].

Any change in the pH of the medium may affect the ionization state of various nutritionally important components and can also reduce their availability for culture cells. Minor decrease in the enzyme production may also be due to the two important factors first, the accumulation of toxic byproducts and secondly the depletion of important nutrients in the medium, which can stop further multiplication of the culture cells responsible for the secretion of enzyme.

Optimization of incubation temperature

Higher level of α-glucosidase production was observed at temperature 35°C and further increase in temperature of the cultures resulted in the decrease in activity of enzyme. Although, a slightly higher enzyme activity observed at 35°C for this enzyme, but this may be due to higher optimum pH of this enzyme. On the other hand, it should be kept in mind that higher biomass obtained in this experiment at 22 °C (fig. 3). In microbes like *Bacillus licheniformis* KIBGE-IB4 and *Lactobacillus acidophilus* maximum activity of α-glucosidase reported at 37 °C [14, 15].

Effect of agitation

The α-Glucosidase activity improved with an increase in agitation speed up to 150 rpm and further increase in agitation gave no noticeable improvement in it. Higher α-glucosidase activity was noticed in cultures incubated at 150 rpm (fig. 4). Growth of protonema of *B. coronatum* was also found maximum at 150 rpm and it slightly decreased with an increase in the rpm. Decrease in enzyme activity may be due to damage to protonemal biomass. Decker and Reski (2007) studied the culture of *Physcomitrella patens* in photo bio reactor (5 l) with 150 rpm in his experiment [16].

Effect of different carbon sources

It was found that sucrose as carbon source was the best source for α-glucosidase production and also for maximum growth of protonemal biomass (fig. 5). After analyzing results, it is suggested that α-glucosidase production is exclusively dependent on the carbon sources used and any variation in the utilization of carbon source can affect the enzyme production. The selective inducer of α-glucosidase for this protonemal culture was sucrose. Similar finding has been reported in *Pseudomonas* sp. SB-15 [17]. The physicochemical characteristics as well as the amylose and amylopectin content of

starch plays an important role for its utilization as carbon source by the plant culture and microbes for α-glucosidase production [18].

Effect of different nitrogen sources

The results presented in fig. 6 indicate that ammonium nitrate is the best nitrogen source, which causes the highest production of α-glucosidase and also maximum growth of protonemal biomass of *B. coronatum.*

Statistical optimization studies

Results of Taguchi experimental design in 16 runs, for the five factors, i.e., sucrose (%), ammonium nitrate (%), temperature, pH, and agitation chosen for optimization of the α-glucosidase productions by protonemal biomass of *B. coronatum* (table 2). Results show the efficiency of the α-glucosidase production that resulted in a range of 3.84-14.39 U/ml corresponding to the combined effect of the five factors in their specific ranges. Taguchi analysis suggests that these factors at optimum level strongly support for enhanced production of α-glucosidase.

The lowest and highest levels of α-glucosidase production were observed in run 1, sucrose (1%), ammonium nitrate (0.5%), temperature (20°C) and agitation (110 rpm), and run 10 with a combination of sucrose (1.5%), ammonium nitrate (0.5%), pH (6.0), temperature (30°C) and agitation (150 rpm), respectively.

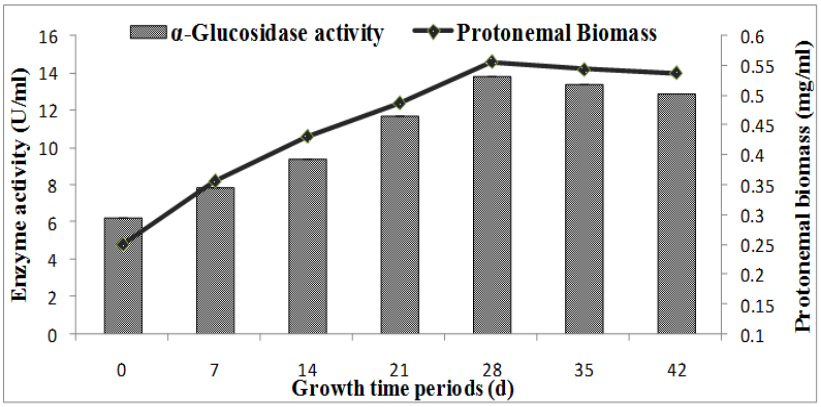

Fig. 1: Effect of growth time on protonemal biomass and α-glucosidase production with respect to growth of protonemal biomass

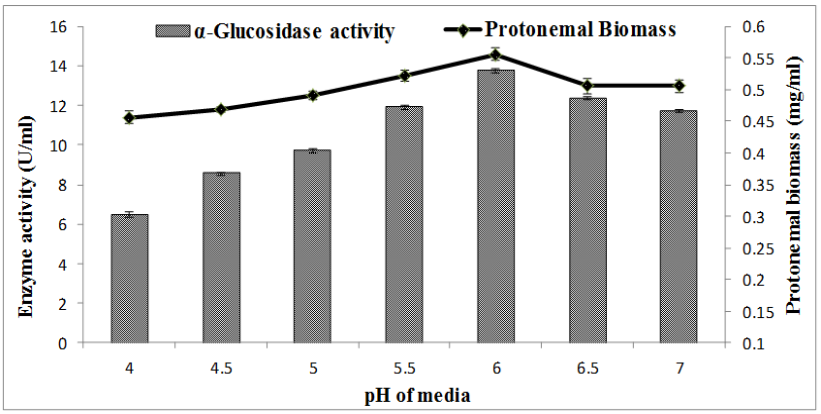

Fig. 2: Effect of pH of the medium on α-glucosidase production with respect to growth of protonemal biomass

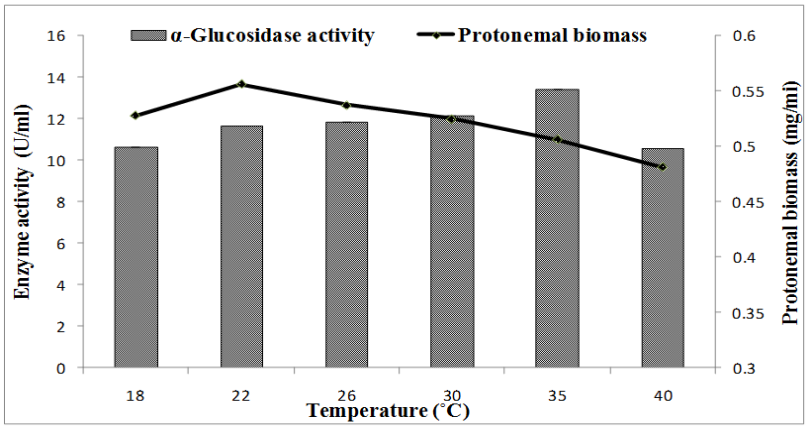

Fig. 3: Effect of incubation temperature on α-glucosidase production with respect to growth of protonemal biomass

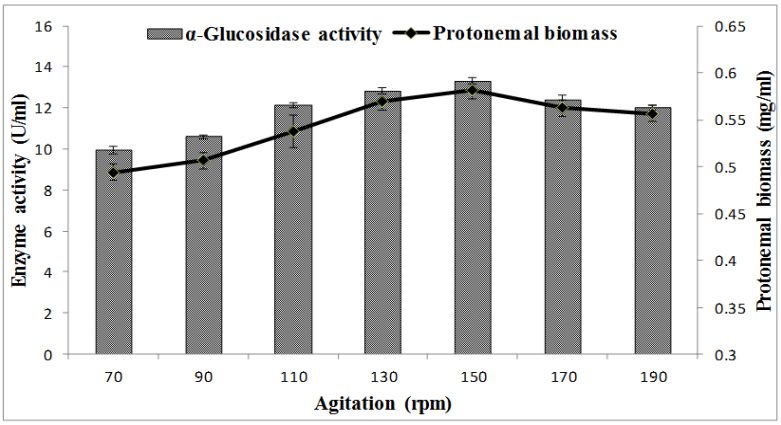

Fig. 4: Effect of agitation on α-glucosidase production with respect to growth of protonemal biomass

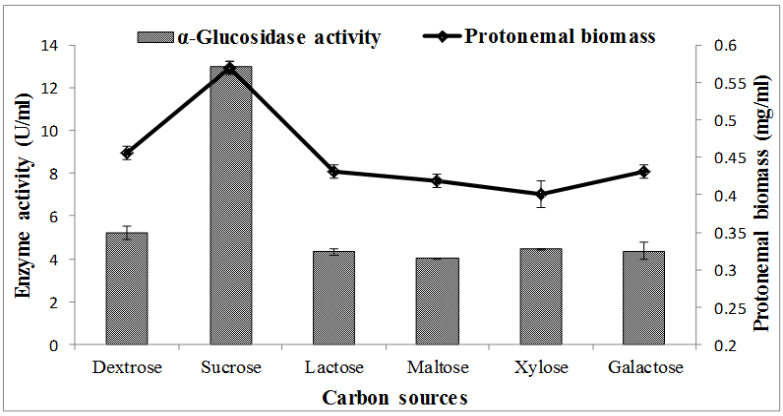

Fig. 5: Effect of different carbon sources on production of α-glucosidase with respect to growth of protonemal biomass

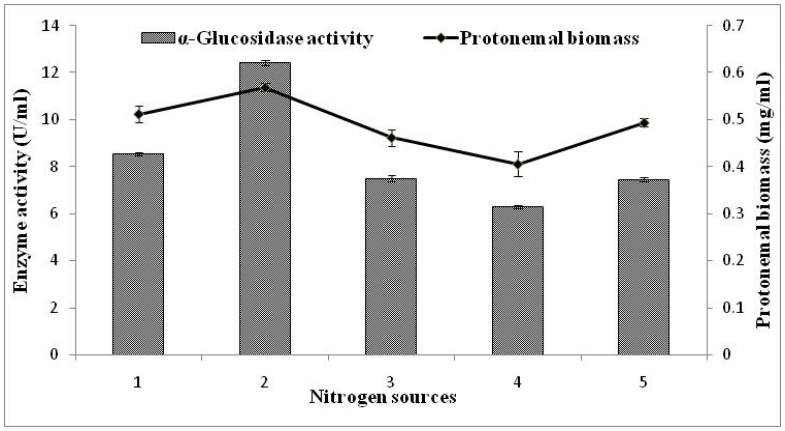

Fig. 6: Effect of different nitrogen sources on production of α-glucosidase with respect to growth of protonemal biomass

Optimum condition and validation experiment based on ANOVA

The significance and percentage contribution of factors on α-glucosidase production variation has been determined from ANOVA (Analysis of variance). From the calculated ratios (F) of all selected parameters, it was noticed that all factors and their interactions considered in the experimental design were statistically significant at 95% confidence limit, indicating that nearly all the variability of experimental data for α-glucosidase production can be explained in terms of significant effects. Taguchi analysis of α-glucosidase production revealed that among all the selected factors, sucrose exhibited the highest significance (28.3%) followed by temperature (22.1%), ammonium nitrate (21.6%) while the agitation (14.3%) and pH (13.4%) showed the least significance on α-glucosidase production under the studied experimental set up (fig. 7 and table 3). These results suggest that α-glucosidase production by the protonema of *B. coronatum* was more influenced by the carbon source (sucrose) rather than nitrogen source (ammonium nitrate).

The error observed was very low which indicated the accuracy of the experimentation. Based on the ANOVA, the software generated the optimum level for each factor and the amount of α-glucosidase production by maintaining the factors at these specified levels. For effective α-glucosidase production, it was inferred that sucrose (1.5%), agitation (150 rpm) at level 3 and ammonium nitrate (1%) and pH (6.0) at level 2 would be optimum, suggesting that physiological conditions and medium ingredients at the respective concentrations have a

significant effect on α-glucosidase production (table 4). To validate the condition, experiments were carried out using these optimized conditions and it was observed that the maximum activity of α-glucosidase was 14.39 U/ml.

Table 2: Taguchi experimental design and corresponding α-glucosidase production by protonemal biomass of *B. coronatum*

Run	Sucrose (1 %)	NH_4NO_3 (0.5 %)	Temperature (ºC)	pH	Agitation (rpm)	α-Glucosidase (U/ml)
1	L1	L1	L1	L1	L1	3.84
2	L1	L2	L2	L2	L2	11.07
3	L1	L3	L3	L3	L3	11.44
4	L1	L4	L4	L4	L4	8.42
5	L2	L1	L2	L3	L4	8.36
6	L2	L2	L1	L4	L3	11.25
7	L2	L3	L4	L1	L2	9.21
8	L2	L4	L3	L2	L1	12.26
9	L3	L1	L3	L4	L2	11.19
10	L3	L2	L4	L3	L1	14.39
11	L3	L3	L1	L2	L4	11.63
12	L3	L3	L2	L1	L3	12.81
13	L4	L1	L4	L2	L3	12.56
14	L4	L2	L3	L1	L4	12.26
15	L4	L3	L2	L4	L1	7.05
16	L4	L4	L1	L3	L2	7.65

Table 3: Analysis of variance of experimental data for α-glucosidase production

S. No.	Factors	DOF	Sums of squares	Variance	F-Ratio	Pure sum	Percent
1	Sucrose (%, w/v)	3	91.99	30.66	1,105.98	91.91	28.27
2	NH_4NO_3 (%, w/v)	3	70.30	23.43	845.21	70.22	21.60
3	Temperature (°C)	3	71.89	23.96	864.32	71.81	22.08
4	pH	3	43.43	14.47	522.18	43.35	13.33
5	Agitation	3	46.57	15.52	559.88	46.48	14.30
	Other/Error	32	0.88	0.027			0.40
	Total:	47	325.09				100.00

Table 4: Optimized condition and their contribution on α-glucosidase production

S. No.	Factor	Level description	Level	Contribution
1	Sucrose (%, w/v)	1.5	3	2.181
2	NH_4NO_3 (%, w/v)	1	2	1.920
3	Temperature (°C)	35	3	1.467
4	Medium pH	6	2	1.501
5	Agitation(rpm)	150	3	1.637
	Total contribution from all factors			8.705
	Current grand average of performance			10.328
	Expected result at optimum condition			19.034

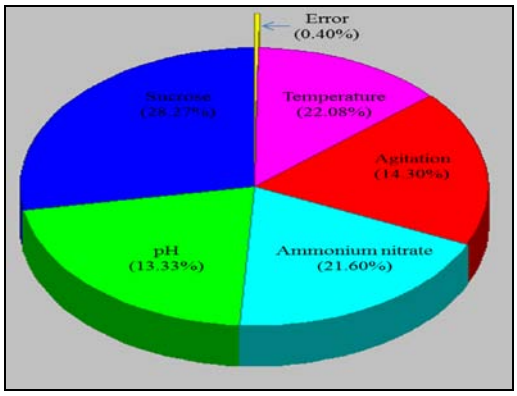

Fig. 7: Contribution of various factors on the α-glucosidase production (%)

CONCLUSION

From the above studies, it can be concluded that the culture of moss *B. coronatum* may be considered as a good source for the α-glucosidase production. Sucrose as carbon source and ammonium nitrate as nitrogen source, temperature 35 °C and pH 6.0 were found to contribute to give optimum for both protonemal biomass growth and enzyme production. Optimization of culture medium by Taguchi method has resulted in an increase in the α-glucosidase activity from 3.84 to 14.39 U/ml.

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CONFLICT OF INTERESTS

Declared None

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