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# Optimal conditions for enhanced β-mannanase production by recombinant *Aspergillus sojae*

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ABSTRACT

Optimization of the growth conditions for maximum β-mannanase production in shake flasks by using recombinant *Aspergillus sojae* ATCC11906 (AsT1) was carried out by Box–Behnken design of response surface methodology. The highest β-mannanase activity on the fourth day of cultivation at 30 °C was obtained as 363 U/ml in the optimized medium consisting of 7% sugar beet molasses, 0.43% $NH_4NO_3$, 0.1% $K_2HPO_4$ and 0.05% $MgSO_4$ (by weight per volume) at 207 rpm. On the sixth day of cultivation under the optimized conditions, the highest β-mannanase activity was achieved as 482 U/ml which is 1.4-fold of 352 U/ml activity found on glucose medium previously.

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## 1. Introduction

β-Mannanases (EC 3.2.1.78) are extracellular enzymes hydrolysing 1,4-β-D-mannosidic linkages in mannans and heteromannans (e.g. galactomannans, glucomannans and galactoglucomannans) which are widely distributed in nature as part of the hemicellulose fraction in hardwoods and softwoods, seeds of leguminous plants and in beans. β-Mannanases have been used in various industries for degradation of thickening agents like guar gum and locust bean gum, production of prebiotics and instant coffee (food industry), enzymatic bleaching (pulp and paper industry), stain removal (detergent industry), diet improvement by hydrolytic action (poultry feed industry), etc.[1,2]. Their role is to facilitate the process steps (e.g. lignin removal, extraction) and improve process quality while reducing the environmental pollution caused by using the chemicals [3–5].

The genus *Aspergillus* is an important industrial microorganism for large-scale production of both homologous and heterologous enzymes (e.g. cellulases and hemicellulases) which have a large potential market [6,7]. *Aspergillus sojae*, which is considered to be safe, is already used in the food fermentation industry to produce fermented food products such as sake, miso and soy sauce [8,9].

In the previous study, because of the pathogenic nature of *Aspergillus fumigatus* IMI 385708, the mature protein coding region of the *A. fumigatus* endo-β-1,4-mannanase gene, *afman*, was isolated from *A. fumigatus* and introduced into *A. sojae*, under the control of the strong glyceraldehyde-3-phosphate dehydrogenase (*gpd*A) promoter from *Aspergillus nidulans*, to attain high level of constitutive β-mannanase production and finally a successful overproduction of the *A. fumigatus* β-mannanase in *A. sojae* was achieved and it was found that *A. sojae* transformant 1 (AsT1) showed the highest activity as 352 U/ml [10].

To meet the growing demands in the industry it is necessary to improve the performance of the system by increasing the yield while decreasing the cost of production. The growth and enzyme production of the organism are strongly influenced by medium composition and external factors (pH, temperature, agitation, etc.); thus optimization of media components and growth parameters is the primary task for enhancement of cost-effective enzyme production in a biological process [11]. In order to achieve this, cheap carbon and nitrogen sources should be selected for the culture media. Sugar beet molasses is the by-product obtained after the final crystallization process in sugar industry. It is used in the production of valuable industrial products like ethanol, pullulan, alpha-amylase and several other enzymes by many microbial sources including fungi [12–19]. In spite of the successful utilization of organic nitrogen sources (e.g. yeast extract, peptone), cheap inorganic salts (e.g. $NH_4NO_3$, $NaNO_3$, $Na_2SO_4$) are also promising media components for fermentation processes [20–22].

Response surface methodology (RSM) has been widely used in optimization studies of several biotechnological and industrial processes by evaluating the interactions of various parameters while reducing time-consumption [23]. β-Mannanase was produced by several *Aspergillus* species [1], but only few optimization studies, which result in low β-mannanase activity, were found in the literature. In this work, recombinant *A. sojae* (AsT1) was used for heterologous production of β-mannanase under the control of *gpd*A promoter and optimization studies for utilization of cheap carbon

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and nitrogen sources (sugar beet molasses and $NH_4NO_3$) as well as the influence of agitation rate on β-mannanase production were carried out for cost-effective production.

## 2. Experimental

### 2.1. Fungal strain

The *A. sojae* (ATCC11906) strain was kindly provided by Dr. Peter Punt from TNO Nutrition and Food Research, Department of Microbiology Holland. Cloning and transformation of endo-β-1,4-mannanase from *A. fumigatus* IMI 385708 into *A. sojae* was formerly carried out in our laboratory and *A. sojae* transformant 1 (AsT1) was found as the best β-mannanase producer among the others [10]. Stock cultures of AsT1 were grown on potato dextrose agar plates at 30 °C and maintained at 4 °C.

### 2.2. Production of β-mannanase in shake flasks

AsT1 was grown on modified YpSs broth medium containing yeast extract (0.4%, w/v), $K_2HPO_4$ (0.1%, w/v), $MgSO_4{\cdot}7H_2O$ (0.05%, w/v) and glucose (2%, w/v) instead of starch, at 155 rpm and 30 °C which is the optimum temperature for *A. sojae* cultivation as used in the other studies [24–26]. For the optimization studies, 100 ml of modified YpSs media containing different concentrations of sugar beet molasses and different concentrations of $NH_4NO_3$ were used for fungal growth at different agitation speeds. After choosing the carbon and nitrogen sources, the effect of initial pH value of growth media on β-mannanase activity was investigated using the pH range of 3.0–8.0. Optimum pH values, whose maximum activity ranged between 7.0 and 8.0 was used in optimization studies. The initial pH values of the media used in optimization studies were measured as 7.2–7.3 [27].

### 2.3. Inoculum preparation

Spore suspensions were prepared by pouring sterile saline–tween solution on top of stock cultures on PDA agar plates and collecting the mixture containing spores of AsT1 in 15 ml sterile falcon tubes. The number of spores were counted using "Thoma Haemocytometer" (Chang Bioscience Inc.).

### 2.4. Enzyme assay

Equal number of spores ($3.6 \times 10^{-6}$ sp/ml) of AsT1 was inoculated into the cultivation media. 1.8 ml of solution containing locust bean gum (0.05%, w/v) in 0.1 M Na–citrate buffer (pH 6.0) was incubated with 0.2 ml of culture supernatant at 50 °C for 5 min. The reaction was stopped by adding 3 ml DNS (3′,5′-dinitrosalicylic acid) solution. To develop the color, total volume was boiled at 90 °C for 15 min and then cooled. Absorbances of the released reducing sugars were measured at 540 nm in UV–Visible Spectrophotometer (PharmaSpec UV-1700, SHIMADZU) [28]. The activity values were expressed as U/ml (Unit/ml supernatant) according to the definition of β-mannanase activity by using mannose standard curve. One unit of enzyme activity (1 U = 16.7 nkat) was defined as the amount of enzyme liberating reducing sugars equivalent to 1 μmol D-mannose in 1 min [29].

### 2.5. Experimental design and statistical analysis

In this study, the optimization of medium components and culture conditions for β-mannanase production by the fungus AsT1 was carried out by using response surface methodology. In preliminary experiments, the effects of various carbon and nitrogen sources and different agitation speeds for maximum β-mannanase production were evaluated by 'one-factor-at-a-time' approach [30]. The best carbon and nitrogen source that resulted in the highest β-mannanase activity was used for optimization by RSM using Box–Behnken design [31]. The ranges of factors studied were as follows: 1–8% (w/v) of molasses, 0.1–0.5% (w/v) of $NH_4NO_3$, and 100–250 rpm of agitation speed. The three factor Box–Behnken experimental plan is given in Table 1. The following second-order polynomial equation was used to develop a predictive model for β-mannanase activity:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC$$

where $Y$ is the predicted response; $\beta_0$ intercept; $\beta_1$, $\beta_2$, $\beta_3$ linear coefficients; $\beta_{11}$, $\beta_{22}$, $\beta_{33}$ squared coefficients; $\beta_{12}$, $\beta_{13}$, $\beta_{23}$ interaction coefficients; $A$, concentration of molasses; $B$, concentration of $NH_4NO_3$; $C$, agitation rate; $A^2$, $B^2$, $C^2$, $AB$, $AC$, $BC$ are levels of the independent variables. MINITAB 13 (Minitab Inc., USA) was used for ANOVA, regression analysis, and generation of surface plots for determining the optimum levels of the variables for maximal production of β-mannanase.

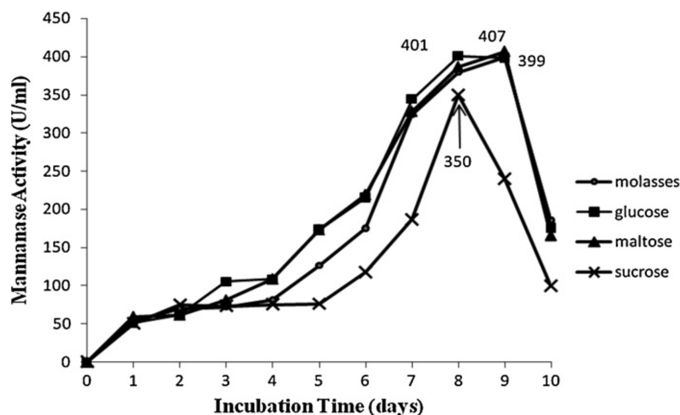


**Fig. 1.** β-Mannanase activity of AsT1 grown on modified YpSs including equal amounts of different carbon sources at 30 °C, 155 rpm.

## 3. Results and discussion

### 3.1. Screening of the factors for optimization studies of β-mannanase production

#### 3.1.1. Effect of different carbon sources

AsT1 was previously grown in 1250 ml of modified YpSs medium containing 2% (w/v) glucose instead of starch. The maximum β-mannanase activity was found as 352 U/ml on the 6th day of cultivation [10,27]. To investigate the effects of alternative carbon sources on β-mannanase activity, 2% (w/v) of maltose, sucrose and sugar beet molasses (containing 2%, w/v carbon), were compared with glucose (Fig. 1). According to ANOVA and Tukey's test results, there was no significant difference in activity values ($P = 0.203 > 0.05$). Since molasses is the by-product of sugar industry, its usage in fermentation processes can also contribute to reduction of environmental pollution and it already finds wide usage area due to its low cost and readily accessable and nutrient rich nature. Thus, to achieve a cost-effective production, molasses was selected as the best and most economical carbon source instead of glucose in modified YpSs broth medium.

#### 3.1.2. Effect of different nitrogen sources

For selection of best nitrogen source alternative to yeast extract in modified YpSs medium, equal amount (0.4%, w/v) of $(NH_4)_2SO_4$,

**Table 1**
Results of a three factor-two replicated Box–Behnken experimental design and β-mannanase production by AsT1.

| Run | Independent variables | | | Response | |
|---|---|---|---|---|---|
| | $C_{molasses}$ (%, w/v) | $C_{NH_4NO_3}$ (%, w/v) | $A_{agitation\ speed}$ (rev/min) | Mannanase activity (U/ml) | |
| 1 | 8 | 0.1 | 175 | 342.955 | 331.974 |
| 2 | 4.5 | 0.1 | 100 | 249.962 | 270.370 |
| 3 | 1 | 0.3 | 250 | 217.777 | 199.402 |
| 4 | 1 | 0.1 | 175 | 195.196 | 176.669 |
| 5 | 4.5 | 0.3 | 175 | 325.019 | 313.891 |
| 6 | 4.5 | 0.5 | 250 | 348.132 | 327.474 |
| 7 | 8 | 0.3 | 250 | 354.678 | 342.560 |
| 8 | 4.5 | 0.3 | 175 | 369.325 | 352.017 |
| 9 | 8 | 0.5 | 175 | 303.659 | 323.529 |
| 10 | 8 | 0.3 | 100 | 329.378 | 306.940 |
| 11 | 1 | 0.3 | 100 | 113.533 | 137.816 |
| 12 | 4.5 | 0.3 | 175 | 339.564 | 321.952 |
| 13 | 1 | 0.5 | 175 | 232.262 | 213.593 |
| 14 | 4.5 | 0.5 | 100 | 255.451 | 237.661 |
| 15 | 4.5 | 0.1 | 250 | 309.372 | 285.996 |

$NH_4NO_3$ and beef extract were used, and incubated at 30 °C for 9 days in a shaker incubator (155 rpm) (Fig. 2). According to ANOVA and Tukey's test results, there was no significant difference between the activities achieved in the media containing yeast extract, beef extract and $NH_4NO_3$, but only the results of $(NH_4)_2SO_4$ medium were significantly lower than the other three N sources ($P = 0.002 < 0.05$). The activity achieved on $NH_4NO_3$ containing medium was as high as the activities achieved on organic ones. Since our aim was an economical production of β-mannanase, $NH_4NO_3$, which has the lowest price, was chosen as the best nitrogen source for further optimization studies.

#### 3.1.3. Effect of agitation speed

After choosing the best carbon and nitrogen sources, the effect of different agitation speeds on β-mannanase activity was investigated. Agitation rate is important for efficient mass transfer of oxygen and nutrients in fungal fermentation and excessively high agitation rates may cause shear stresses on fungal mycelia, thus it has a critical effect on fungal mycelia formation during growth [32,33]. The results revealed that the activity increased when the agitation rate was increased (Fig. 3). According to ANOVA and Tukey's test results, the highest agitation speed (250 rpm) resulted in significantly higher activity values than the others ($P = 0.006 < 0.05$). Since the mycelia structure of the fungus was destroyed at agitation rates higher than 250 rpm, an appropriate range of agitation speed for optimization studies was selected as 100–250 rpm.

### 3.2. Optimization studies by response surface methodology

#### 3.2.1. Box–Behnken RSM design

The concentrations of both carbon and nitrogen sources in the growth media are important in order to eliminate the possible repression and inhibition effects of the compounds. β-Mannanase activities in different concentrations of molasses (1–10%, w/v) and $NH_4NO_3$ (0.1–0.5%, w/v) and at different agitation rates (100–250 rpm) were analysed (Table 1). The Box–Behnken experimental plan with three factors and three levels including six replicates at the centre point was used for fitting the second-order response surface [34]. A total of 30 runs of experiments were carried out in two replicates (Table 1).

A multiple regression analysis of the data was carried out for obtaining an empirical model that relates the response (β-mannanase activity) measured to the independent variables (molasses, $NH_4NO_3$, agitation). The data obtained for β-mannanase activity from 30 experimental points were used for optimizing the medium components. Confidence interval for statistical analysis was 95%, and $\alpha = 0.05$. The regression coefficients for each term are given in Table 2. The second-order polynomial equation for the β-mannanase activity was found to be:

$$\beta\text{-mannanase activity (U/ml)} = -165.1 + 75.2M + 241.056N + 2.417A - 4.4MM - 458.6NN - 0.006AA - 21.7MN - 0.05MA + 0.9NA$$

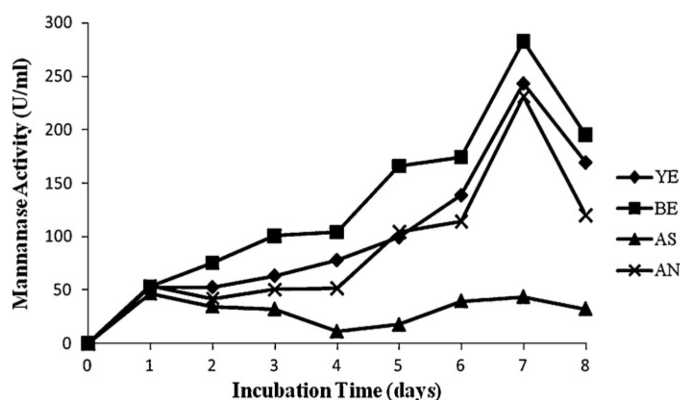


**Fig. 2.** β-Mannanase activity of AsT1 grown on modified YpSs including equal amounts of different nitrogen sources at 30 °C, 155 rpm: yeast extract (YE), beef extract (BE), $(NH_4)_2SO_4$ (AS) and $NH_4NO_3$ (AN).

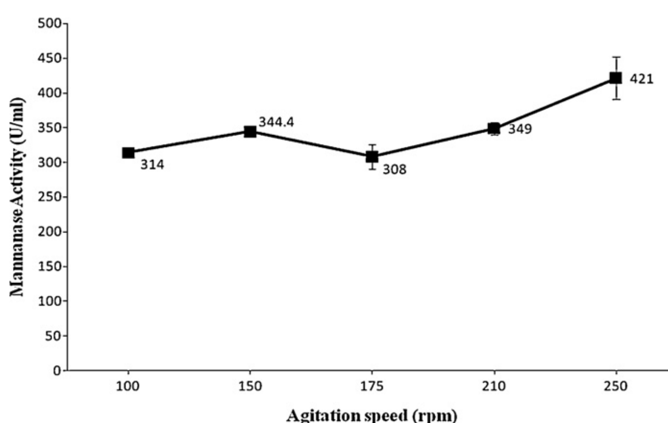


**Fig. 3.** β-Mannanase activities in the modified YpSs medium (w/v); molasses (2.24% sucrose), 0.43% $NH_4NO_3$ at 30 °C, at different agitation rates.

**Table 2**
Response surface regression (confidence interval of 95%).

| Term | Coefficient | SE coefficient | $T$ | $P<0.05$ |
|---|---|---|---|---|
| Constant | −165.1 | 50.954 | −3.240 | 0.004 |
| Molasses | 75.2 | 9.566 | 10.481 | 0.000 |
| $NH_4NO_3$ | 241.1 | 133.445 | 1.806 | 0.086 |
| Agitation | 2.4 | 0.452 | 5.347 | 0.000 |
| Molasses × molasses | −4.4 | 0.968 | −8.042 | 0.000 |
| $NH_4NO_3 \times NH_4NO_3$ | −458.6 | 166.738 | −2.751 | 0.012 |
| Agitation × agitation | −0.006 | 0.001 | −4.958 | 0.000 |
| Molasses × $NH_4NO_3$ | −21.7 | 12.205 | −2.375 | 0.028 |
| Molasses × agitation | −0.05 | 0.033 | −2.047 | 0.054 |
| $NH_4NO_3$ × agitation | 0.9 | 0.427 | 2.096 | 0.049 |

$S=18.12$; **R-Sq = 95.2%**; R-Sq (adj) = 93.1%.

**Table 3**
Analysis of variance for β-mannanase activity (ANOVA).

| Source | DF | Seq SS | Adj SS | Adj MS | $F$ | $P$ |
|---|---|---|---|---|---|---|
| Regression | 9 | 130,864 | 130,864 | 14540.4 | 44.27 | 0.000 |
| Linear | 3 | 97,624 | 39,931 | 13310.3 | 40.52 | 0.000 |
| Square | 3 | 28,568 | 28,568 | 9522.7 | 28.99 | 0.000 |
| Interaction | 3 | 4671 | 4671 | 1557.2 | 4.74 | 0.012 |
| Residual error | 20 | 6570 | 6570 | 328.5 | | |
| **Lack of fit** | 3 | 2143 | 2143 | 714.4 | 2.74 | **0.075** |
| Pure error | 17 | 4426 | 4426 | 260.4 | | |
| Total | 29 | 137,434 | | | | |

where $M$, molasses concentration; $N$, $NH_4NO_3$ concentration; $A$, agitation speed. The coefficient of determination, $R^2$, was calculated to be 0.952 for β-mannanase activity. The $R^2$ value is a measure of how much variability in observed values can be explained by the factors and their interactions. Therefore; it can be concluded that a good quadratic model was obtained. The adjusted $R^2$ (93.1%) value was very close to the predicted $R^2$ (95.2%) value. The 'lack of fit $P$-value' 0.075 > 0.05 for β-mannanase activity implied that the lack of fit is insignificant and the model was adequate (Table 3). This indicated a good agreement between experimental and predicted values.

The effects of the individual factors and their interactions on β-mannanase activity are shown by response surface plots (Fig. 4a–c). In Fig. 4a, it can be seen that β-mannanase activity increases when the concentrations of carbon and nitrogen sources increase and it levels off for high amounts of carbon while gradually decreases for high amounts of nitrogen sources. In Fig. 4b, maximum activity values were obtained close to the high values of agitation speed (175–250 rpm) and molasses concentrations (6–8%, w/v) which cover the optimum values of molasses and agitation rate (207 rpm and 7%, w/v). In Fig. 4c, effects of $NH_4NO_3$ concentration and agitation speed are shown. In this plot, under an agitation speed of 175 rpm, the concentration of nitrogen source does not significantly affect the β-mannanase activity and an increase in the agitation speed positively influences the activity. A maximum activity was obtained when the agitation speed is in the range of 175–250 rpm and the concentration of nitrogen source is between 0.3 and 0.5% (w/v).

The optimum medium components predicted by the model had the composition: 7% (w/v) molasses (2.24% sucrose), 0.43% (w/v) $NH_4NO_3$, 0.1% (w/v) $K_2HPO_4$, and 0.05% (w/v) $MgSO_4$. The maximum activity was then calculated as 356 U/ml (5945 nkat/ml) when AsT1 was grown at 207 rpm on 4th day of cultivation.

There are some studies in the literature in which β-mannanase was produced by different microorganisms grown on different media. In 2001, Setati et al. [35] cloned and expressed the β-mannanase gene of *Aspergillus aculeatus* in *Saccharomyces cerevisiae* and 521 nkat/ml of maximum activity was achieved after the growth in complex media. Ademark et al. [36] studied β-mannanase production by *Aspergillus niger* grown in locust bean gum medium (inducer for β-mannanase secretion) and the activity was found as 90 nkat/ml. Among the few optimization studies for cost-effective β-mannanase production, El-Helow et al. [20] studied a $2^n$ factorial design (CCD) to optimize the culture conditions for β-mannanase production by *Bacillus subtilis* cultivated on the media containing cheap agro-industrial by-products, wheat bran and palm seed powder. The highest β-mannanase activity was achieved as 102 U/ml under the optimized medium containing palm seed powder. Regalado et al. [37] used RSM to maximize the production of β-mannanase from solid state fermentation of soluble coffee wastes using *Aspergillus oryzae*. The maximum activity was found as 108 mg mannose $min^{-1} L^{-1}$ (∼0.6 U/ml). Kote et al. [38] investigated the effects of culture conditions and medium components for optimizing β-mannanase production by *A. niger*. They found that β-mannanase activity was influenced by the type of carbon source. Highest activity (40 U/ml) was achieved on copra meal defatted with $n$-hexane. In these studies, agro-industrial

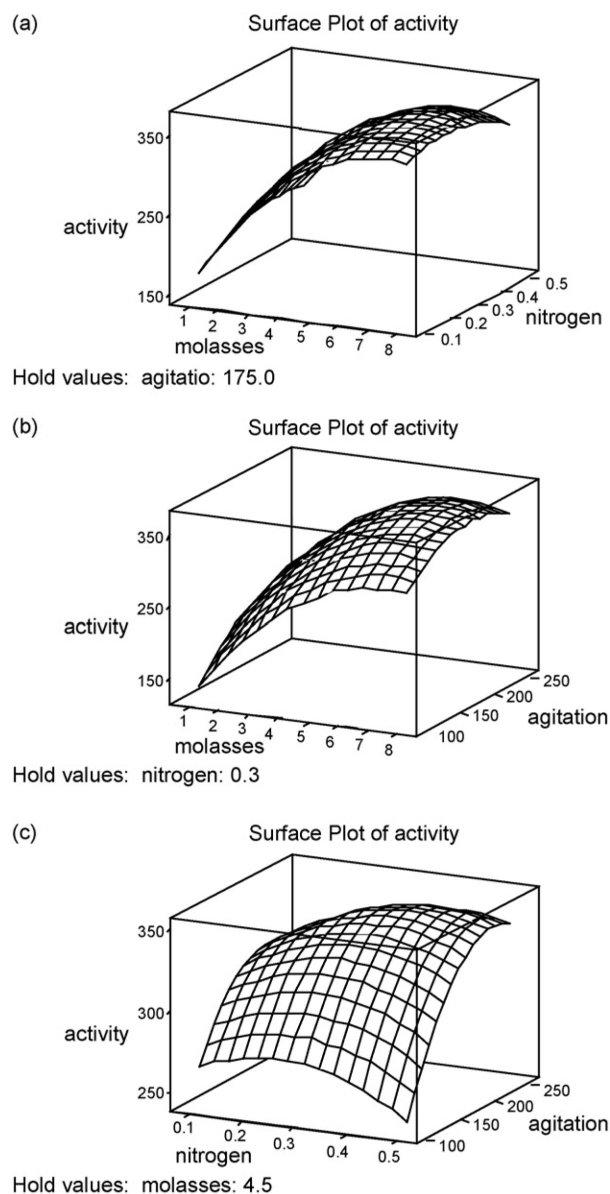


**Fig. 4.** (a) Response surface curves of β-mannanase production as a function of molasses and nitrogen concentrations (%, w/v). (b) Response surface curves of β-mannanase production as a function of molasses concentration (%, w/v) and agitation speed (rpm). (c) Response surface curves of β-mannanase production as a function of nitrogen concentration (%, w/v) and agitation speed (rpm).

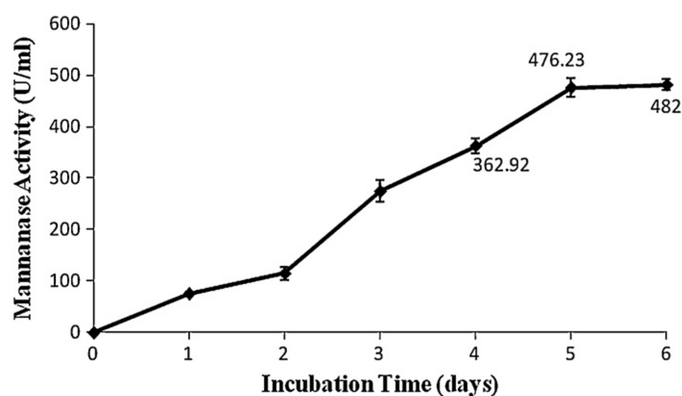


**Fig. 5.** Time course of β-mannanase production by AsT1 under the predicted optimum conditions; modified YpSs medium (7% molasses, 0.43% $NH_4NO_3$, 0.1% $K_2HPO_4$, 0.05% $MgSO_4$, w/v) at 30 °C, 207 rpm.

wastes/by-products were used with many supplemental salts and minerals, however the β-mannanase activities achieved were very low for industrial applications. To our knowledge, the maximum β-mannanase activity achieved in our study is by far the highest compared to the β-mannanases and recombinant β-mannanases of other fungi in the literature.

### 3.2.2. *Validation of the optimum condition defined by the model*

The quadratic model was validated by conducting experiments under the optimum conditions predicted by the model. The average of the activities obtained from 5 replicates was 362.92 ± 13.9 U/ml on the fourth day of incubation. This was very close to the value estimated by the model (356 U/ml). Thus it can be concluded that the constructed model can be successfully used to predict the β-mannanase production under various combinations of molasses, $NH_4NO_3$, and agitation. The incubation was continued until the 6th day and the maximum activity (482 U/ml = 8050 nkat/ml) was achieved on the 6th day of cultivation (Fig. 5).

## 4. Conclusion

Our findings suggest that among the various alternative carbon and nitrogen sources tested in this study, sugar beet molasses and $NH_4NO_3$ were by far the best sources for the economical production of β-mannanase in recombinant *A. sojae*. Response surface methodology was found to be effective in optimizing the β-mannanase production by *A. sojae*. The optimum conditions for maximum β-mannanase production were achieved when AsT1 was grown in the modified YpSs medium containing 7% (w/v) molasses (2.24%, w/v sucrose), 0.43% (w/v) $NH_4NO_3$ and at 207 rpm of agitation rate according to RSM using Box–Behnken design. Thus, β-mannanase activity achieved by optimizing the culture conditions and medium components (482 U/ml) was 37% higher than the activity found on glucose medium (352 U/ml). Such a high and cost-effective production of endo-β-1,4-mannanase enzyme by an industrially safe fungus *A. sojae* can be effectively used in reducing the viscosity of coffee extract to improve the process technology in instant coffee production. In poultry feed industry, for diet improvement, highly active enzymes (e.g. β-mannanases) are needed to keep the enzyme/feed ratio (w/w) as small as possible. Finally, utilization of molasses provides a low cost, nutrient rich source to the fermentation industry for large-scale production of value added products such as enzymes.

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