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ENHANCED β-MANNANASE PRODUCTION FROM ALTERNATIVE SOURCES BY RECOMBINANT *ASPERGILLUS SOJAE*

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β-mannanases can degrade galactomannans to mannose and it has been used in various application areas. The aim of this study was to produce the β-mannanase from carob pod extract including different nitrogen sources. The best operation values for fermentation were determined to be 8% initial sugar concentration with 0.5% yeast extract, 100 r.p.m., and 7% inoculation rate, which yielded the maximum β-mannanase activity as 423.60 U ml^{-1}. Effects of nitrogen sources on β-mannanase activity were also studied and it reached 695.6 U ml^{-1} by using 0.5% of ammonium nitrate as the nitrogen source at the determined optimum conditions. Results also showed that meat bone meal and soybean meal could be used as cost effective nitrogen sources based on achieved β-mannanase activity.

Keywords: β-mannanase, recombinant *Aspergillus sojae*, carbon sources, nitrogen sources

Microbial mannanase has gained significant attention, since it degrades the complex polysaccharides of plant tissues into simple small molecules such as manno-oligosaccharides and mannose. For example, the increased availability and high specificity of β-mannanases have been used for numerous applications, such as production of manno-oligosaccharides, pharmaceutical applications, instant coffee production, animal feeds, paper and pulp production, detergent formulations etc. (Van Zyl et al., 2010; Lu et al., 2014).

β-Mannanase could be generally produced by various *Aspergillus* species (Van Zyl et al., 2010) and the activity of the produced enzymes can differ from species to species. Also, other microorganisms have been evaluated for β-mannanase productions. Chen and co-workers (2007) expressed *Aspergillus sulphures* β-mannanase gene in *Pichia pastoris* and the specific activity was found to be 366 U ml^{-1} in locust bean gum. Ozturk and co-workers (2010) used recombinant *Aspergillus sojae* ATCC11906 (AsT1) for β-mannanase production from molasses and found the highest activity value as 363 U ml^{-1}. Wang and co-workers (2010) have found that *Pantoea agglomerans* A021 could produce β-mannanase from konjac powder and the maximum activity of purified Man26P was 514 U mg^{-1}. Wu and co-workers (2011) used *Aspergillus niger* E-30 to produce the β-mannanase from peeled potato and the maximum β-mannanase activity value was 1067.5 U mg^{-1} by purified enzyme solution

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towards locust bean gum. *Aspergillus niger* CBS 513.88 gene was expressed in *Pichia pastoris* for mannanase production, and maximum mannanase activity was 430.9 U mg^{-1} from the culture supernatant including yeast extract, peptone, and glucose as nitrogen and carbon source (Zhao et al., 2011). It has been also reported that β-mannanase activity was found 513.3 U ml^{-1} by using corn steep liquor dextrose medium by recombinant *Pichia pastoris* (Zheng et al., 2012). Yin and co-workers (2012a) used *Paenibacillus cookii* to produce mannanase using a media including 0.1% locust bean gum with 1.5% soytone and 0.5% tryptone, and the highest specific activity for crude and purified enzymes were 7.0 U mg^{-1} and 635.4 U mg^{-1}, respectively. And the other studies for mannanase were 310.1 U ml^{-1} by *Bacillus* sp. N16-5 using the modified alkaline Horikoshi-II medium (Lin et al., 2007), 3.78 U ml^{-1} by recombinant *Escherichia coli* in Luria-Bertani medium including 50 μg ml^{-1} kanamycin (Katrolia et al., 2013), 1122 U mg^{-1} by recombinant *Pichia pastoris* in yeast-peptone-dextrose medium (Luo et al., 2012), 1231.41 U ml^{-1} by *Bacillus subtilis* Bs5 from konjac powder (Huang et al., 2012), and 561.3 U g^{-1} by *Aspergillus niger* SN-09 from apple pomace (Yin et al., 2012b).

In the literature, the β-mannanase production has been performed using cheap alternative sources such as sugar beet molasses (Ozturk et al., 2010), coffee waste, and wheat bran (Kurakake & Komaki, 2001) in place of pure carbon sources for cost-effective production, and hence carob can also be used as a cheap alternative source for production of value-added products. Carob (*Ceratonia siliqua* L.) is an evergreen tree widely grown in Mediterranean countries: Spain, Portugal, Morocco, Greece, and Turkey etc. In the food industry, carob is used to produce locust bean gum, a process that only necessitates the seed part of carob pods (~10% by weight) and the residuary (90%) is used only to make a traditional carob concentrate called "pekmez" in Turkish (Karkacier et al., 1995). When its fruit is ripe enough to be harvested, it has 91–92% total dry matter and 62-67% total soluble solids, which consist of 34–42% sucrose, 10–12% fructose, and 7–10% glucose. With its rich medium content, carob is known to be utilized by biotechnological methods, especially fermentation (Turhan et al., 2010a; 2010b). Purified sugars in medium increase yield, but the cost also increases. Therefore, researchers have evaluated alternative complex carbon sources, such as molasses (Ozturk et al., 2010) and apple pomace, in order to decrease the fermentation costs (Yin et al., 2012b).

There is no study on mannanase production from carob pod extract including alternative nitrogen sources. Therefore, this study is undertaken to produce β-mannanase by using genetically modified *Aspergillus sojae* in alternative carbon source medium (carob pod extract) and to optimize it in terms of initial sugar concentration (%), agitation speed (r.p.m.), and inoculation rate (%) by using response surface methodology (RSM) as well as various nitrogen sources.

1. Materials and methods

In this study, recombinant *Aspergillus sojae* transformant 1 (AsT1) (Duruksu et al., 2009) was used, which was obtained from Prof. Dr. Z.B. Ogel's laboratory (Middle East Technical University, Ankara, Turkey). The stock culture was grown on potato dextrose agar (PDA) at 30 ºC for 4–5 days, stored at 4 ºC, and sub-cultured bi-monthly. Inoculum was prepared by washing each incubated PDA agar plate with 5 ml of sterile saline (0.8%, w/v) and Tween-80 (0.005%, v/v) mixed solution (Duruksu et al., 2009; Ozturk et al., 2010). The resulting suspension solution had about 4.0×10^7 spores per ml.

1.1. Fermentation

For β-mannanase fermentation studies, carob pod extract at various sugar levels was supplemented with 0.4% (w/v) yeast extract, 0.05% $MgSO_4.7H_2O$, and 0.1% K_2HPO_4 per liter, and called as carob pod extract medium (CPEM) (Ozturk et al., 2010). For the flask optimization studies, 100 ml of CPEM was used with different sugar concentrations, spore levels, and agitation rates. Samples were taken every day for 10 days and stored at 4 ºC until analysed. The chopped carob pods without seeds were stored at 4 ºC until extraction. Carob pod extractions were carried out using 1:4 solid:liquid ratio (carob:water ratio by weight) at 80 ºC for 2 h in a water bath (Turhan et al., 2010a).

1.2. Experimental design for CPEM fermentations

β-Mannanase production with CPEM was optimized by Box-Behnken response surface method (Box & Behnken, 1960). In this study, three different fermentation factors were selected: initial sugar concentration (X_1) (1–8%) (Ozturk et al., 2010), agitation (X_2) (100–250 r.p.m.), and inoculation rate (X_3) (5–7%). Minitab (Version 15.0. Minitab Inc. State College, PA, USA) statistical software was used to determine the best conditions for β-mannanase production and for the analysis of variance (ANOVA) and regression analysis to illustrate the coefficients of the model and significant terms. Finally, the model was verified by performing fermentation at the suggested optimum condition. During the optimized fermentation, different amounts (0.5, 0.75, and 1%) of pure [yeast extract (YE), beef extract (BE), and ammonium nitrate (NH_4NO_3)] and industrial vegetal or animal by-products (Red lentil, cotton-seed meal, maize zein, meat-bone meal, feather meal, and fish meal) were used to determine the best nitrogen source (Ozturk et al., 2010).

1.3. Analysis

Samples were taken every day for 10 days and analysed for biomass, enzyme activity, and residual sugar in the fermentation broth. Fermented broth was firstly filtered by filter paper (Whatman No: 1) and dried at 60 ºC for 24 h to measure weight of dry biomass (Ozturk, 2008). β-Mannanase activity values were calculated as U ml^{-1} (Unit ml^{-1} supernatant) using mannose standard curve [Y=3.9424×X–0.3632, where Y is mannose concentration (μM ml^{-1}) and X is absorbance at 575 nm] by DNS method from supernatant of fermented broth by spectrophotometer (Shimadzu UV-160A, Japan). One unit of enzyme was defined as the amount of enzyme releasing reducing sugars equivalent to 1 μmol D-mannose in per minute at 50 ºC (Ozturk et al., 2010). Residual sugar content in fermentation broth was determined by DNSA method (Miller, 1959). Absorbance values were converted to residual sugar concentration by using the standard curve [Y=70.011×X+0.495, where Y is sucrose concentration (g l^{-1}) and X is absorbance at 575 nm].

2. Results and discussion

This study was designed to optimize culture conditions as well as evaluation of alternative nitrogen sources for mannanase production.

2.1. Optimization of fermentation conditions

Initial sugar levels (1, 4.5, and 8%), agitation rates (100, 175, and 250 r.p.m.), and inoculum size (5, 6, and 7%) were optimized for fermentation conditions by *Aspergillus sojae* (AsT1) in CPEM and β-mannanase activity results are given in Table 1.

Table 1. β-Mannanase production results for two replicated Box-Behnken experimental design

No	Initial sugar concentration (%)	Agitation (r.p.m.)	Inoculation rate (%)	β-Mannanase activity (U ml^{-1})		Biomass growth (g l^{-1})
				Experimental	Predicted	
1	8	250	6	284.99	302.26	12.66
2	4.50	175	6	201.13	205.64	10.96
3	4.50	100	7	251.33	244.73	6.05
4	1	250	6	182.88	179.51	3.70
5	4.50	100	5	183.58	142.89	8.49
6	8	175	5	357.10	307.87	15.90
7	4.50	250	7	279.89	264.71	8.36
8	4.50	250	5	205.40	255.40	7.52
9	1	100	6	127.09	125.84	4.22
10	1	175	5	186.66	167.93	5.07
11	4.50	175	6	179.99	153.41	9.51
12	8	100	6	331.92	246.06	4.93
13	4.50	175	6	215.88	208.65	14.37
14	1	175	7	165.53	154.62	4.20
15	8	175	7	352.51	290.57	15.44

When comparing the results, it was clearly seen that higher initial sugar concentrations gave higher β-mannanase production (from 246.06 U ml^{-1} to 357.10 U ml^{-1}, mean: 309.16 U ml^{-1}). The highest activity value (357.10 U ml^{-1}) was obtained at 8% initial sugar concentration (Table 2 and Fig. 1). On the other hand, the lowest activity was measured as 125.84 U ml^{-1}, when 1% initial sugar concentration was used in medium (Fig. 1). Consequently, the regression analysis results showed that the effect of initial sugar concentration was significant ($P<0.05$) on β-mannanase production (Table 2).

Table 2. Regression coefficients for β-mannanase production from carob pod extract

Term	Coefficients	P-value
Constant	194.12	0.000*
Initial sugar conc. (X_1)	73.95	0.000*
Agitation (X_2)	18.85	0.023*
Inoculation rate (X_3)	12.32	0.124
$X_1 \times X_1$	23.90	0.047*
$X_2 \times X_2$	4.55	0.691
$X_3 \times X_3$	29.83	0.016*
$X_1 \times X_2$	–12.52	0.262
$X_1 \times X_3$	1.57	0.886
$X_2 \times X_3$	–10.72	0.335

*$P<0.05$ is significant and $R^2=0.851$. R^2 (adj)=0.784

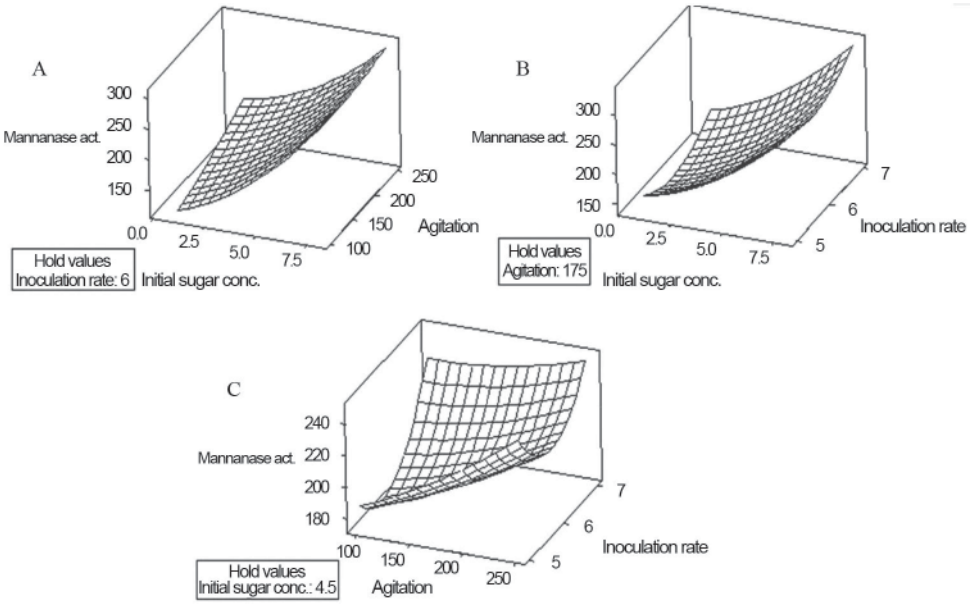

Fig. 1. Response surface plots (A: The effect of initial sugar conc. and agitation, B: The effect of initial sugar conc. and inoculation rate, C: The effect of agitation and inoculation rate)

Choosing the right agitation rate is crucial to provide good mass and heat transfer during the entire fungal fermentation. For this purpose, different agitation rates (100, 175, and 200 r.p.m.) were evaluated to determine the optimum setting. The results showed that the highest β-mannanase production values were obtained at 175 r.p.m. (two of the highest: 352.51 U ml^{-1} and 357.10 U ml^{-1}) (Table 1 and Fig. 1) and there were significant differences ($P<0.05$) between the agitation levels and β-mannanase activity (Table 2).

Three different inoculation rates (5, 6, and 7%) were performed to find out how the initial inoculum concentrations affect the whole fermentation process. The highest mannanase activity values were achieved 352.51 and 357.10 U ml^{-1} at 7 and 5% inoculation rates, respectively (Table 1 and Fig. 1), and the regression analysis results showed that it was significant ($P<0.05$) for β-mannanase production (Table 2).

β-Mannanase activity values were used as response to carry out a multiple regression analysis of the data and get a model that relates the response. After regression analysis (Table 2), the data fitted into a second order polynomial equation and β-mannanase activity model curve was found to be:

$$Y=194.12+73.95X_1+18.85X_2+23.9X_1^2+29.83X_3^2 \quad (1)$$

where Y is the predicted β-mannanase concentration.

Our results showed that the model was good reliable ($R^2=0.851$, $P<0.05$) (Table 2) and according to the ANOVA results the non-significant lack of fit value ($P=0.054>0.05$) demonstrates that the model fit well to experimental data of β-mannanase activity. It was found

from the model equation that initial sugar conc., agitation, initial sugar conc.× initial sugar conc., and inoculation rate × inoculation rate were significant ($P<0.05$) parameters (Table 2).

Two optimal working conditions were found from the RSM optimizer. The first one (Opt. 1) was 8% initial sugar concentration, 147 r.p.m., 7% inoculation rate; and the second one (Opt. 2) was 8% initial sugar concentration, 100 r.p.m., 7% inoculation rate. Two fermentation runs were performed at the determined optimal conditions to validate the equation of the β-mannanase model and the results are given in Table 3. The values estimated by the model were 337.96 and 344.63 U ml^{-1} and the values determined by assays were 339.66 (Fig. 2A) and 350.82 U ml^{-1} (Fig. 2B), respectively, and it can be obviously concluded that the model can be successfully used to predict the β-mannanase activity values under various combinations of carob pod extract initial sugar concentration, agitation, and inoculation rate with 0.4% yeast extract. Under optimized conditions with different fermentation runs, the highest β-mannanase activity was obtained to be 423.60 U ml^{-1} with 0.5% yeast extract. These results were higher than the ones reported (363 U ml^{-1}) by Ozturk and co-workers (2010) using molasses as a carbon source. Similarly, Soni and co-workers (2015) optimized the production of mannanase from palm kernel cake (PKC) as substrate by using *Aspergillus terrus* FBCC 1369 via RSM, and the effect of particle size of substrate, pH, moisture content, and carbon and nitrogen supplements was studied. Optimized medium consisted of 5 g PKC of 0.5 mm particle size, LBG 1% (w/v), and urea 1% (w/v) as carbon and nitrogen supplementation with 12.8 ml moisture content, with a pH of pH 8.0, which resulted in a maximum yield of 417 U gds^{-1} β-mannanase. On the other hand, El-Sharouny and co-workers (2015) also studied the optimization of the fermentation medium components using Placket-Burman design, and glucose and inoculum size were found as the most important factors enhancing the production of enzyme. Using optimized medium in the fermentation process, enzyme activity of 42.2 U ml^{-1} was achieved.

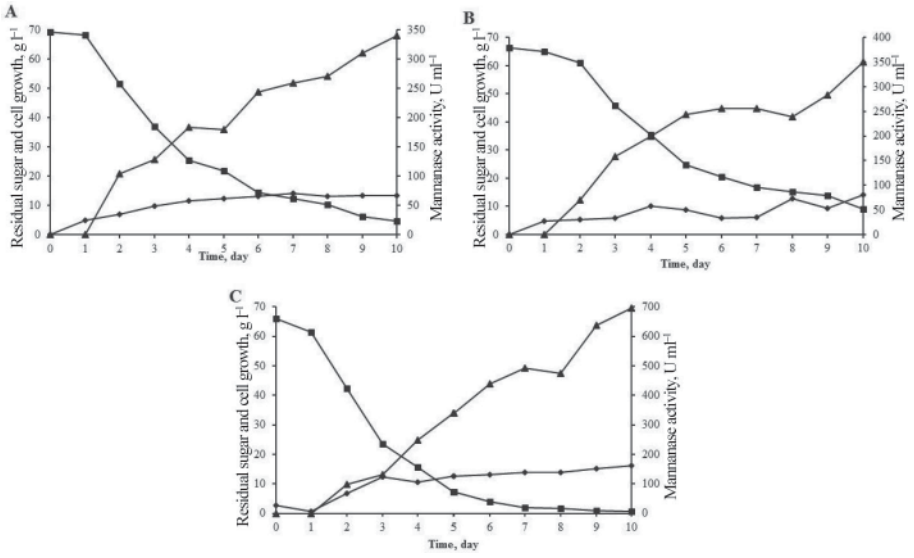

Fig. 2. Fermentation plots belonging to optimized conditions and highest β-mannanase activity (A: Opt. 1, B: Opt. 2, and C: The plot belong to highest mannanase production obtained using medium including 0.5% AN)
—■—: residual sugar; —◆—: cell growth; —▲—: β-mannanase activity

Table 3. Kinetic parameters of β-mannanase production at optimum conditions

Kinetic parameters	Opt. 1	Opt. 2
Sugar consumed (g l^{-1})	64.73	57.51
Biomass growth (g l^{-1})	14.16	14.12
β-Mannanase activity (U ml^{-1} day^{-1})	339.66*	350.82*
Max. consumption rate (–ds/dt)max (g l^{-1} day^{-1})	8.00	7.52
Max. production rate (dp/dt)max (U ml^{-1} day^{-1})	29.03	26.48

* β-Mannanase activity predicted value calculated from model is 337.96 and 344.63 U ml^{-1}

Biomass concentration is affected by fermentation conditions (Table 1). It is changed by agitation speed and initial sugar concentration, but not by inoculation rate. The biomass concentration is increasing with the increasing initial sugar concentration, but is reduced at minimum and maximum agitation speeds. In conclusion, high mannanase activities are obtained at high biomass concentrations.

2.2. Effect of different nitrogen sources

In order to enhance the β-mannanase activity from carob pod extract with optimized conditions, different levels (0.5, 0.75, and 1%) of pure [yeast extract (YE), beef extract (BE), and ammonium nitrate (AN - NH_4NO_3)] and industrial vegetal or animal by-products (Red lentil, cotton-seed meal, maize zein, meat-bone meal, feather meal, and fish meal) were used as nitrogen source in CPEM. Results showed that the maximum β-mannanase activity, 695.6 U ml^{-1}, was obtained at 0.5% AN (Fig. 2C), while the lowest β-mannanase activity was 185.7 U ml^{-1} with 1% of AN (Table 4). But AN is a pure and expensive material for fermentation. Comparing results of the animal and vegetal by-products, meat bone meal and soybean meal gave the highest β-mannanase activities (597.31 and 525.93 U ml^{-1} with 0.75% concentration, respectively). These results indicate that meat bone and soybean meals were good alternative nitrogen sources for β-mannanase production from CPEM. A negative correlation between β-mannanase activity and amount of pure nitrogen sources was found, meaning that higher amount of pure nitrogen source in fermentation media resulted in lower β-mannanase activity. But this was not true for animal and vegetative nitrogen sources.

Similarly, Dhawan and co-workers (2015) investigated the effect of inexpensive agro-residues (apple pomace, orange peel, potato peel, copra meal, oat bran, and wheat bran) on β-mannanase production by using *Paenibacillus thiaminollyticus*. According to their results, the β-mannanase activities were found as 50, 55, 80, 372, 527, and 590 U ml^{-1}, respectively. They have also studied the effect of different nitrogen sources (ammonium sulphate, yeast extract, peptone, and their combinations) on β-mannanase production by using *Paenibacillus thiaminollyticus* and the mannanase activities were determined as 643.5, 625, 581, and 670 U ml^{-1}, respectively. In conclusion, to date in the literature, β-mannanase production was was studied in flasks and bioreactor assays with different microorganisms, different medium and nitrogen sources. The maximum β-mannanase activity obtained by us was the highest available in the literature until now without any separation or purification process.

Table 4. Mannanase activities in different nitrogen sources

Source type	Nitrogen source	Mannanase activity (U ml^{-1})		
		0.5%	0.75%	1%
Pure	Ammonium nitrate	695.60±34.73	311.50±0.57	185.70±0.76
	Yeast extract	423.60±41.39	304.80±43.68	278.40±39.00
	Beef extract	472.00±34.65	351.68±71.81	317.60±26.96
Animal by-products	Meat-bone meal	530.77±15.8	597.31±10.18	315.79±1.67
	Feather meal	231.02±1.80	319.50±2.86	355.77±5.37
	Fish meal	277.4±5.61	267.82±13.19	310.74±7.91
Vegetal by-products	Red lentil	228.23±0.36	217.91±25.45	247.11±6.63
	Cotton-seed meal	202.40±3.69	279.82±14.06	315.75±28.30
	Maize zein	203.37±10.07	200.33±41.20	228.17±6.59
	Soybean meal	402.81±33.23	525.93±29.96	445.02±8.48

3. Conclusions

In this study, carob pod extract was chosen for cost-effective carbon source, and culture growth conditions were optimized by RSM. Different nitrogen sources were studied to enhance β-mannanase activity from carob pod extract under optimized conditions. Fermentation conditions were optimized by RSM, and the highest β-mannanase activity found was 423.60 U ml^{-1}. Then, under optimized conditions, the β-mannanase activity was enhanced to 695.6 U ml^{-1} by using 0.5% of AN in fermentation medium without any separation and purification process. According to our experiments, vegetal or animal by-products could also be used as nitrogen source. Comparing the results with the literature shows that carob pod extract (carbon source), meat bone meal (nitrogen source), and soybean meal (nitrogen source) can be alternatively used for β-mannanase production.

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