OPTIMIZATION OF NUTRIENT COMPOSITION MEDIUM AND CULTURE CONDITION FOR MANNANASE PRODUCTION BY *BACILLUS VELEZENSIS* NRC-1 USING TAGUCHI METHOD

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ABSTRACT

The effect of different nutrient composition media on the production of mannanase by *Bacillus velezensis* NRC-1 was investigated. Medium 1 (composed of (g/L), peptone, 2.0; $(NH_4)_2SO_4$, 1.5; $MgSO_4 \cdot 7H_2O$, 0.5; K_2HPO_4, 10.0; locust bean gum, 10.0, urea and 0.3, pH 5.3) was found to be the most favorable medium for mannanase production which recorded, 2.19 U/mL, after 7 days of incubation at 30 ºC. Taguchi orthogonal array method was used for optimization of nutrient composition medium and culture condition for mannanase production. Eight nutrient factors were studied including peptone, ammonium sulphate, urea, magnesium sulphate, dihydrogen potassium phosphate, locust bean gum, pH and fermentation volume with three levels. Analysis of variance (ANOVA) revealed that peptone concentration, locust bean gum and pH of the medium were the most influencing factors with percent participation 19.25, 13.46 and 57.89%, respectively. An increase in mannanase production to 8.7 U/mL could be achived after the optimization process.

Keywords: *Bacillus velezensis*, Taguchi method, Mannanase

1. INTRODUCTION

Endo-β-1,4-mannanase (EC 3.2.1.78) is a crucial enzyme for the depolymerization of unsubstituted mannans, galactomannans and galacto-glucomannans **(Stalbrand *et al.*, 1993; de Vries and Visser, 2001)**. The mannanases are widely used in food, instant coffee processing, paper and pulp industries **(Wong and Saddler, 1993; Montiel *et al.*, 1999; Sachslehner *et al.*, 2000*;* Ferreira and Filho, 2004; Gübitz *et al.*, 1997)**. Recently β-1,4-mannanases have been shown to be effective in laundry detergents **(McCoy, 2001 and Schäfer *et al.*, 2002)**. Mannanase is also used in poultry feed industry to break down the mannan found in poultry feeds to oligosaccharides and reduce intestinal viscosity which then improves feed efficiency and increase poultry growth **(lee *et al.*, 2003 and Wu *et al.*, 2005)**.

Mannanases are produced from bacteria as *Bacillus subtilis* (Mendoza *et al*., 1995), *Streptomyces lividans* **(Arcand *et al.*, 1993)** and *Vibrio* species **(Tamaru *et al.*, 1995)**; fungi as *Aspergillus* species, *Trichoderma reesei* **(Stalbrand *et al.*, 1995)**, *Penicillium occitanis* and plants as *lycopersicon esculentum* **(Bewley *et al*., 1997)**.

The Taguchi method has been recently used for the optimization of production of many enzymes as xylanses **(Azin *et al*., 2007)** and laccases **(Prasad *et al.*, 2005)**

In this study, the effect of some medium composition, pH and fermentation volume on mannanase biosynthesis by *Bacillus velezensis* NRC-1 using Taguchi method was conducted.

2. MATERIALS AND METHODS

2.1 Microorganism

The microorganism used in this study, *Bacillus velezensis* NRC-1 was isolated locally from dehaired skin of sheep. The identification of *Bacillus velezensis* NRC-1 was carried out using partial sequencing of ribosomal DNA gene, with phylogenetic analysis.

2.2 Chemicals

All chemicals were of pure grade produced by known manufacturers.

2.3. Culture media

The medium used for inoculating and maintenance of *B. velezensis* NRC-1 composed of (g/L) peptone, 5; beef extract, 3.0; NaCl, 8.0 and agar 12.0. For growth enhancement tryptone liquid nutrient medium was used (g/L) tryptone, 10.0; yeast extract, 5.0 and NaCl, 10.0.

2.4. Production media

Different types of nutrient production media of various compositions were used to select the most suitable medium for the production of mannanase. The types of media and composition are listed below. Ingredients of each medium were dissolved in 1 L of distilled water. The prepared nutrient medium was distributed in 100 mL portions in triplicate, each transferred into 250 mL conical flasks plugged with cotton and sterilized by autoclaving at 121 °C and 1.5 atm for 20 min. Some components of the nutrient media such as sugar were sterilized separately and then added aseptically to the media.

Medium 1. (g/L), peptone, 2.0; $(NH_4)_2SO_4$, 1.5; $MgSO_4 \cdot 7H_2O$, 0.5; K_2HPO_4, 10.0; locust bean gum, 10.0; urea 0.3, and the pH was adjusted to 5.3 **(Arisan-Atac *et al*., 1993)**.

Medium 2. (g/100ml) (mannose submerged fermentation)

Locust bean gum, 0.5; Na_2 HPO_4, 0.7; K_2 HPO_4, 3.0; NH_4Cl, 0.1; NaCl, 0.05 and the pH was adjusted at 8 **(Bhoria *et al*., 2009)**.

Medium 3. (g/L)

Guar gum, 5.0; Na_2HPO_4, 7.0; KH_2PO_4, 3.0; NH_4Cl, 1.0; NaCl, 0.5 and the pH was adjusted at 8 **(Bhoria *et al*., 2009)**.

Medium 4. (g/L)

Glycerol, 20.0; meat peptone, 20.0; KH_2PO_4, 1.2; $MgSO_4.7H_2O$, 1.5; KCl, 0.6; NH_4NO_2, 0.5 and traces of metal solution in a level 0.3 ml/L **(Sachslehner *et al*., 1998)**.

Medium 5. (%)

Konjac powder, 1.0; Yeast extract, 2.0; Polypeptone, 2.0; KH_2PO_4, 0.1; $MgSO_4.7H_2O$, 0.02 and $NaCO_3$ 0.5 **(Akino *et al*., 1987)**.

Medium 6. (g/100ml)

Locust bean gum, 1.0; Polypeptone, 3.0; $MgSO_4.7H_2O$, 0.06; KH_2PO_4, 1.5; Corn steep liquor, 2.5 and the pH was adjusted at 7 **(Abe *et al*., 1994)**.

Medium 7. (g/100ml)

Konjac powder, 2.0; Na_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.6; beef peptone, 0.8 and yeast extract 0.4 **(Jiang *et al*., 2006)**.

Medium 8. (g/L)

Konjac powder, 6.0; meat peptone, 6.0; corn steep liquor, 1.0; $(NH_4)_2SO_4$, 1.0; Na_2HPO_4, 0.8; K H_2PO_4, 0.06; $MgCl_2$, 0.12; $CaCl_2$, 0.6; $FeSO_4$, 0.002 and Na_2CO_3, 0.6 **(Feng *et al*., 2003)**.

Medium 9. (g/L)

Starch insoluble, 6.0; Na_2HPO_4,7.0; KH_2PO_4, 3.0; NH_4Cl_2,1.0; NaCl, 0.5 and the pH was adjusted at 8 **(Bhoria *et al*., 2009)**.

2.5. Enzyme assay :

The culture extract of the fermentation medium was centrifuged at 10,000×g for 15 min. The supernatant was used as the source of enzymes. Mannanase activity was determined by measuring total reducing sugars released from 1% (w/v) locust bean gum as a substrate in 1 mL citrate buffer, 50 mM, pH 5, using 0.5 mL of the growth broth containing mannanase enzyme was added. The mixture was incubated at 50 °C for 10 min and the enzyme activity was determined by the method of Somogyi **(Somogyi, 1945)**. One unit of β-mannanase activity was defined as the amount of enzyme which releases 1 mol of reducing sugar as equivalent to D-mannose per minute under the above mentioned conditions.

2.6. Protein determination :

The concentration of soluble proteins was estimated according to the method of **Lowry *et al*., (1951)** using bovine serum albumin (BSA) as the standard reference.

3. EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS:

Taguchi method was used for optimization of medium composition and culture conditions to produce mannanase enzyme. Standard orthogonal array L-27 (3^8) was selected to examine eight-factors (peptone, ammonium sulphate, urea, magnesium sulphate, dihydrogen potassium phosphate, locust bean gum, pH and volume) with three levels. The factors were selected on basis of standard fermentation conditions on medium No.1, with changes in some physical conditions as pH and volume, to measure their effect on mannanase biosynthesis from *B. velezensis* NRC-1. The symbolic array L-27 of experimental matrix represents the size of experiment and the number of runs (i.e. 27 experimental trails) with layout (3^8). The three levels of the eight factors were coded as 1, 2 and 3 (Table 1). The total degree of freedom (DOF) for OA L-27 set was 26 (number of runs minus one). In the design OA, each column consisted of a number of conditions depending on the levels assigned to each factor. The runs involved a particular combination of levels to which the factors were set, and the diversity of factors was studied by crossing the factors. The whole experiment was performed in triplicate manner. An analysis of variance (ANOVA) for the obtained results was investigated. The incubation of culture medium was carried out at 30 ºC for 7 days in all Taguchi runs.

Table 1. Selected fermentation factors and their assigned levels for optimization process for *B. velezensis*.

Serial no.	Factor	Level 1	Level 2	Level 3
1	Peptone (g/L)	3.5	2.0	0.5
2	Ammonium sulphate (g/L)	1.5	1.0	0.2
3	Urea (g/L)	0.30	0.10	0.05
4	Magnesium sulphate (g/L)	0.50	0.20	0.05
5	Potassium dihydrogen phosphate (g/L)	15	10	5
6	Locust bean gum (g/L)	15	10	5
7	Volume (mL)	50	25	15
8	pH	8.0	6.5	5.3

4. RESULTS

A preliminary experiment was conducted to select the most suitable nutrient medium for mannanase biosynthesis by *B. velezensis*. Nine nutrient media with varying compositions were selected from previous studies carried out on mannanase biosynthesis with different incubation periods (3, 5 and 7 days). Medium 1 was found to be the most suitable medium with mannanase biosynthesis of 2.19 U/mL after 7 days incubation (Table 2).

Table 2. Effect of different culture nutrient media during different incubation periods on the biosynthesis of mannanase by *Bacillus velezensis* NRC-1.

Media No.	Incubation period (days)	Dry weight of cells (g)	pH	Protein content (mg/mL)	Mannanase activity (U/mL)	Specific activity ($U.mg^{-1}$ protein)
1	3	0.50	6.5	1.80	1.20	0.67
	5	0.54	6.0	2.56	1.55	0.61
	7	0.73	6.5	2.09	2.19	1.05
2	3	1.20	6.0	1.06	0.85	0.80
	5	1.13	5.5	1.46	1.23	0.84
	7	1.98	6.0	1.5	1.57	1.05
3	3	0.7	8.0	0.73	0.10	0.13
	5	0.64	7.0	1.38	0.14	0.10
	7	0.85	7.5	1.81	0.13	0.07
4	3	2.4	5.5	3.92	0.10	0.02
	5	2.32	7.5	6.53	0.13	0.02
	7	1.89	8.5	6.16	-	-
5	3	3.0	9.0	4.29	2.12	0.49
	5	2.89	9.0	10.5	2.11	0.20
	7	2.81	9.0	11.3	1.54	0.14
6	3	2.4	7.0	3.84	1.44	0.38
	5	2.23	8.0	8.25	1.95	0.24
	7	1.04	8.5	9.04	1.76	0.19
7	3	1.12	8.0	2.33	1.64	0.70
	5	1.07	7.5	4.11	1.93	0.47
	7	0.60	7.5	4.04	2.06	0.63
8	3	0.63	8.5	1.74	1.23	0.71
	5	0.61	7.0	2.13	1.29	0.61
	7	0.72	8.5	2.02	1.95	0.97
9	3	0.90	7.0	1.03	0.16	0.16
	5	0.85	6.0	1.24	0.21	0.17
	7	0.84	7.0	1.22	0.35	0.29

As a result medium 1 was selected for optimization process using Taguchi method. By using Taguchi method standard orthogonal array L-27 (3^8) eight-factors (peptone, ammonium sulphate, urea, magnesium sulphate, dihydrogen potassium phosphate, locust bean gum, pH and volume) with three levels from each was selected to study their effect on mannanase production (Table 3). The results of the experiments performed revealed that the maximum production of mannanase was 7.95 U/mL under the following medium composition (g/L) peptone, 3.5; ammonium sulphate, 1.50; urea, 0.30; magnesium sulphate, 0.20; potassium dihydrogen phosphate, 10.0; locust bean gum, 10.0 with fermentation volume, 25 mL and pH 6.5. Analysis of variance (ANOVA) of the obtained results (Table 4) revealed that changing of peptone and locust bean gum in medium composition together with pH have been the most important factors in causing differences in the obtained results.

When these results were analyzed, an optimum condition could be attained by calculations as follows, medium composition (g/L) peptone, 3.5; ammonium sulphate, 1.50; urea, 0.30; magnesium sulphate, 0.05; potassium dihydrogen phosphate, 12.0,; locust bean gum, 15.0 with fermentation volume, 25 mL and pH 6.5. Under these conditions, mannanase biosynthesis was expected to be 8.9 U/mL. However, after performing the experiment with the above mentioned conditions, the produced mannanse was found to be 8.7 U/mL. Since the difference between the expected and actual is 2.25%, the result was regarded as acceptable.

Table 3. Taguchi method orthogonal array L-27 (3^8) of designed experiments.

Run	Factor								Mannanase activity (U/mL)	D.W.* (g/L)
	1	2	3	4	5	6	7	8		
1	2.0	1.5	0.05	0.20	5	15	25	5.3	1.93	0.53
2	0.5	0.2	0.30	0.05	15	5	25	5.3	2.07	0.73
3	0.5	1.0	0.05	0.05	5	10	50	8.0	6.11	0.84
4	0.5	0.2	0.30	0.50	10	15	15	8.0	4.31	0.4
5	3.5	0.2	0.05	0.50	5	5	15	6.5	6.43	0.26
6	2.0	1.0	0.30	0.20	15	10	15	6.5	7.48	0.57
7	2.0	0.2	0.10	0.20	10	5	50	8.0	3.33	0.94
8	0.5	1.0	0.05	0.20	10	15	15	5.3	3.51	0.47
9	2.0	1.5	0.05	0.05	15	10	15	8.0	5.83	0.65
10	3.5	0.2	0.05	0.05	10	10	25	8.0	4.98	0.72
11	3.5	1.5	0.30	0.20	10	10	25	6.5	7.95	0.73
12	2.0	0.2	0.10	0.50	15	10	15	5.3	4.29	0.48
13	2.0	0.2	0.10	0.05	5	15	25	6.5	6.21	0.52
14	3.5	1.0	0.10	0.50	10	10	25	5.3	4.85	0.69
15	2.0	1.0	0.30	0.50	5	15	25	8.0	6.33	0.5
16	2.0	1.5	0.05	0.50	10	5	50	6.5	3.92	0.72
17	3.5	1.0	0.10	0.05	15	15	50	6.5	7.96	1.62
18	0.5	1.5	0.10	0.50	5	10	50	5.3	1.45	0.73
19	3.5	1.5	0.30	0.50	15	15	50	8.0	6.69	1.87
20	3.5	0.2	0.05	0.20	15	15	50	5.3	3.57	1.57
21	0.5	1.0	0.05	0.50	15	5	25	6.5	4.49	0.67
22	0.5	0.2	0.30	0.20	5	10	50	6.5	5.13	0.77
23	3.5	1.5	0.30	0.05	5	5	15	5.3	5.09	0.29
24	0.5	1.5	0.10	0.05	10	15	15	6.5	7.00	0.52
25	0.5	1.5	0.10	0.20	15	5	25	8.0	2.10	0.77
26	3.5	1.0	0.10	0.20	5	5	15	8.0	4.31	0.27
27	2.0	1.0	0.30	0.05	10	5	50	5.3	2.68	1.01

*D.W. : Dry weight

Table 4. Analysis of variance of main effects of factors

Factor	Degree of freedom	Sum of squares	F* value	Participation percentage
Peptone (g/L)	1	13.26	11.36	19.25
$(NH_4)_2SO_4$ (g/L)	1	0.38	0.32	0.53
Urea (g/L)	1	3.52	2.69	4.55
$MgSO_4.7H_2O$ (g/L)	1	4.18	0.65	1.11
KH_2PO_4 (g/L)	1	0.23	0.10	0.17
Locust bean gum (g/L)	1	13.26	7.94	13.46
Volume (mL)	1	4.03	1.77	3.01
pH	1	40.96	17.08	57.89

*F: Degree of freedom.

DISCUSSION

Most optimization process used for mannanase production deals with one factor at a time **(Youssef *et al*., 2006)** or using response surface methodology **(Dan *et al*., 2012).** However using Taguchi method enabled us to study eight different factors with three levels affecting mannanase production. According to the results produced, Taguchi method was found useful in mannnase production optimization as it increased production from 2.13 to 8.7 U/mL compared to other methods used. As a result, enzyme production was finally increased about 408 %, in relation to the initial step.

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دراسة الدرجة المثلى للظروف البيئية وتركيباتها لإنتاج إنزيم المانانيز بواسطة بكتيرة *Bacillus velzensis* NRC-1

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قسم كيمياء المنتجات الطبيعية والميكروبية ، المركز القومى للبحوث ، الدقى

قسم النبات والميكروبيولوجى ، كلية علوم (بنين) ، جامعة الأزهر

تمت دراسة تأثير البيئات المختلفة ذات التركيب الغذائى لإنتاج إنزيم المانانييز من سلالة بكتيرية (*Bacillus velezensis* NRC-1) و كانت البيئة الغذائية رقم واحد (و التى تحتوى على جم\لتر : ٢ بيبتون ، ١.٥ امونيوم سالفات ، ٠.٥ سالفات الماغنسيوم ، ١٠ ثنائى البوتاسيم هيدروجين الفوسفات ، ١٠ صمغ حبوب الخروب ، ٠.٣ يوريا عند درجة حموضة ٥.٣) وجد انها البيئة الأكثر ملائمة لإنتاج إنزيم المانانييز و التى سجلت اعلى كفاءة عند ٢.٢٩ وحدة\مللى بعد سبعة ايام من التحضين عند ٣٠ درجة مئوية. و لقد تم استخدام تصميم تاجوشى للوصول الى احسن تركيز غذائى على ثلاث مستويات من كل محتوى. و لقد استخدمنا ٨ عوامل فى هذا التصميم و هم كالآتى : بيبتون ، أمونيوم سالفات ، سالفات الماغنسيوم ، ثنائى بوتاسيم هيدروجين الفوسفات ، صمغ حبوب الخروب ، يوريا ، حجم البيئة الغذائية و درجة الحموضة. و لقد كشف تحليل التباين (ANOVA) ان تركيز البيبتون و صمغ حبوب الخروب و درجة الحموضة من اهم العوامل المؤثرة فى الإنتاج (%) ١٩.٢٥ ، ١٣.٤٦ ، ٥٧.٨٩ . بالتالى يمكن ان يتحقق زيادة فى الإنتاج الى ٨.٧ وحدة\مللى بعد عملية التحسين.