OPTIMIZATION OF AGITATION AND INCUBATION PERIOD ON PRODUCTION OF MANNANASE BY *BACILLUS VELEZENSIS* NRC-1 USING BENCH-SCALE BIOREACTOR.

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ABSTRACT

The effect of agitation speed and incubation period on the production of mannanase enzyme by *Bacillus velezensis* NRC-1 using bench-scale bioreactor at 45ºC was investigated. Results revealed that the increase in agitation speed from 200 to 800 rpm resulted in a significant increase in mannanase production to 21.04 U/mL. The increase in agitation speed affected dissolved oxygen (DO) concentration which in turn affected cell growth and mannanase production. The maximum mannanase production in the bioreactor was attained after 72 h of incubation at 45 ºC. Mannanase activity in the bioreactor (21.04 U/mL) while much higher than that obtained from the shake flask fermentation (15.6 U/mL) also the incubation period decreased from 7 days in shake flask fermentation to 3 days in bioreactor.

Keywords: *Bacillus velezensis*, Mannanase, shaking, bioreactor

1. INTRODUCTION

Beta-mannanase (Endo-1,4-β-D-mannanase, EC 3.2.1.78) is a crucial enzyme that catalyzes the random cleavage of β-D-1,4 mannopyranosyl linkages within the main chain of galactomannan, glucomannan, galactoglucomannan and mannan **(Stoll *et al.*, 1999**). Mannanases have been reported from different organisms; bacteria **(Mendoza *et al.*, 1995)**, fungi **(Arcand *et al.*, 1993)**, higher plants (Bewley *et al*., 1997) and animals **(Yamaura and Matsumoto, 1993)**. β-Mannanases have numerous applications in the food, feed, as well as pulp and paper industries **(Godfrey, 1983; Wong *et al.*, 1993)**. β-Mannanase can be mass cultivated in industrial fermenters by using batch fermentation process. On a commercial scale, the objective is to reduce the duration of fermentation cycle, as a shorter cycle length would minimize energy cost and maximize production output. For full-scale production system, it is essential to devise a scale-up strategy using bioreactor that would adopt desired level of agitation and aeration rates (in the fermentor), which in turn would give comparable or better yields relative to those obtained from shake flask study. This is necessary as it would enable one to minimize production cost and optimize the cost-effectiveness for the overall production process **(Feng *et al.*, 2003)**. Although much work has been done on screening of high β-mannanase producing microorganisms, these efforts have primarily been confined to culture medium and conditions in shake flask studies **(Akino *et al.*, 1987; Ratto and Poutanen, 1988 and Araujo and Ward, 1990)**. Little information is known about optimization conditions on the bench scale fermentor. The objective of this study is to investigate the desired combination of agitation, and incubation period that would yield the highest β-mannanase production by *B. velezensis* nrc-1 in fermenter scale.

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* was done using partial sequencing of ribosomal DNA gene, with phylogenetic analysis.

2.2 Chemicals

All chemicals were of pure grade made by known manufacturers.

2.3. Inoculum medium (Tryptone liquid medium)

The medium used for inoculation of bacterial cultures composed of (g/L): tryptone 10.0, yeast extract 5.0**,** NaCl 10.0. The seed culture was prepared by cultivation of 500 mL of fermentation medium for 24 h at 30 ºC at 150 rpm. This medium was seeded into the 5 L fermentation medium.

2.4. Production medium

Production medium **(Arison- atac *et al.*, 1993)** composed of (g/L), peptone 3.5, $(NH_4)_2SO_4$ 1.5, $MgSO_4 \cdot 7H_2O$ 0.05, K_2HPO_4 12.0, locust bean gum 15.0 and urea 0.3. Five litre fermentation medium was autoclaved on 121 ºC under 1.5 atm for 20 min on stainless steel head plate vessel of the bioreactor. The pH was adjusted using phosphate buffer at 6.5.

2.5 Mannanase production in stirred tank bioreactor

2.5.1 Batch cultivation with respect to agitation speed

The cultivation was carried out in 7 L bioreactor, New Brunswick Scientific Bioflo & celligen 310, Fermentor/bioreactor**.** The vessel was designed for total volume of 7.5 L and working volume of 5 L. It consists of a stainless steel head plate. Ports were provided in the head plate for: inoculation, base and acid addition; a thermo well for a resistance temperature detector; a foam probe; a sparger; a harvest tube; a sampling tube; an exhaust condenser; dissolved oxygen and pH electrodes. On the other hand, the stirrer of the Bioflow III bioreactor was equipped with two 4-bladed-rushton turbines. The agitation speed was carried out at 200, 400, 600 and 800 rpm. Air was introduced into the medium through the ring sparger and the flow rate was controlled by a needle valve located at the control cabinet. Oxygen percentage introduced into the medium was determined manually by the user. One hundred percent or (1.0 v/v. min) of air can be applied through two flow meter, one outside the control cabinet and with manual control while the other one (0-20 SLPM mass flow meter) was inside and automatically controlled. The bioreactor was supplied with inlet filter 0.2 µm interchangeable cartridge.

2.5.2 Sampling system

The system has a sampler which was attached to a sampling tube extending to the lower portion of the vessel at constant time intervals. The sampler has a rubber suction bulb to facilitate collection of representative samples without contamination. A 25 mL screw cap container serves as a reservoir. In every sample, mannanase activity, protein content and cell dry weight were determined.

2.5.3 Determination of cell dry weight

From each sample, only 5 mL were taken and filtered through the Whatman filter paper (20) and then dried at 70 ºC for a constant weight. The weight of the dried cells was

measured by calculation of the difference between the weight of filter paper before and after filtration.

2.5.4 pH control

To observe the manner of pH during the batch, a glass electrode (ingold) was used.

2.5.5 Temperature control

Temperature was controlled at 45 ºC and was sensed by a platinum RTD electrode. Control was maintained by Prim and then by a PID controller which is employed with heater and cooling water.

2.5.6 Dissolved Oxygen (DO)

Dissolved oxygen was calibrated in the range of 0-100%. DO was sensed by a polarographic (in-gold) electrode.

2.6. 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 mannanase enzyme. The 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, when 0.5 mL of mannanase enzyme was added. A standard curve of D-mannose was used as reference. The mixture was incubated at 50 °C for 10 min. 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.

2.7. Protein determination

The concentration of soluble proteins was determined according to the method of **Lowry, Rosebrough, Farr, and Randall (1951)** using bovine serum albumin (BSA) as the standard reference.

3. RESULTS AND DISCUSSION

The production of mannanase enzyme using *B. velezensis* NRC-1 on shake flask level was studied. The maximum mannanase activity produced by *B. velezensis* NRC-1 was found to be 15.6 U/mL at incubation temperature 45 ºC for 7 days at 200 rpm on the production medium (mentioned in materials and methods section). Scale up was done on bench scale bioreactor using the above mentioned conditions and the effect of agitation speed and incubation period was studied.

3.1.Effect of agitation speed

The fermentations were carried out at constant temperature of 45 ºC and aeration rate of 0.5 vvm using different agitation speeds of 200, 400, 600, 800 rpm, respectively (fig. 1). The β-mannanase activity increased almost at the same rate under the agitation speeds 200 and 400 rpm until it reaches its maximum (8.55 and 12.00 U/mL, respectively) after 120 h of incubation. While at agitation speed 600 rpm, β-mannanase activity increased vigorously with high speed during the first 40 h of incubation and then production was almost stable until it reached its maximum after 102 h of incubation (16.85 U/mL).

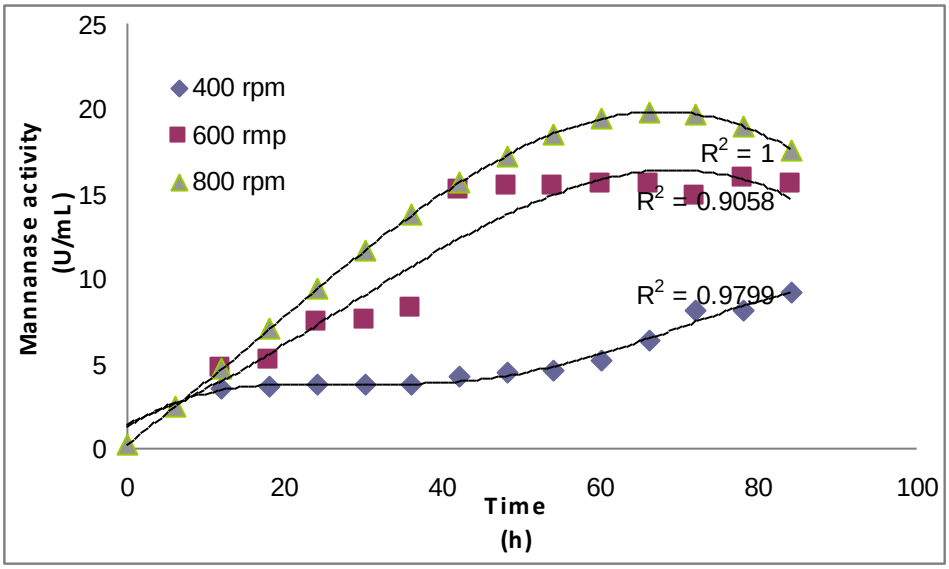

Fig. 1: Relation between time and mannanase activity at different agitation rate.

The maximum production of β-mannanse enzyme was observed at agitation speed 800 rpm. It reaches its maximum production rate after 72 h with activity 21.04 U/mL.

By increasing the agitation speed, it was found that the activity increased considerably. This observation shows that the increase in agitation speed affects the process as the agitation is related to the dissolved oxygen (DO) concentration in the liquid which conclude that the oxygen is an important factor in the process as to be considered a limiting substrate. The dissolved oxygen concentration profiles were different for the four levels of agitation speeds tested. Dissolved oxygen concentrations could be maintained at above 40% saturation over the entire fermentation process at agitation speeds 600 and 800 rpm. However, it was notably lower at 200 and 400 rpm and reached below 20% saturation after 20 h incubation. The oxygen limitation resulted in a decrease in the growth rate and hence in the enzyme activity. It was reported that there was a critical dissolved oxygen concentration below which metabolism pathways might be changed (Atkinson and Mavituna, 1983, Amanullah *et al*., 1998, Okada and Iwamatu, 1997). Also, from the results it was found that the increase in activity about 233% with the increase in rpm from 400 to 600 at 48 h. While the increase of agitation speed from 600 to 800 rpm at 48 h resulted in about 13% increase in mannanase activity. This result strongly suggested the optimum agitation speed to be 800 rpm.

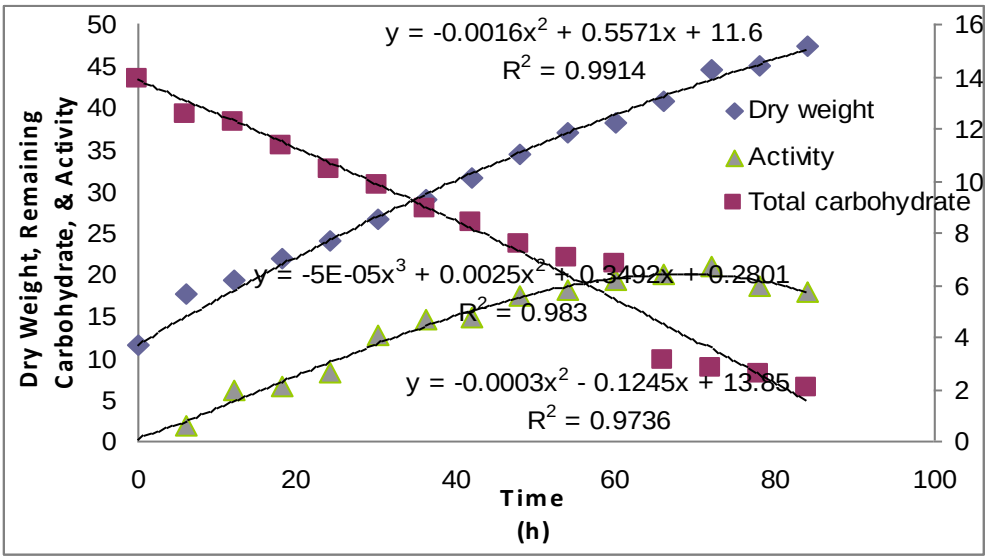

Fig. 2: Relation between time with each of dry weight, remaining carbohydrate and mannanase activity.

Experiment results were fitted by equations to eliminate any errors. The range of R2 indicates the efficiency of the equation selected (0.9-1.0) this mean that as long as the growth rate increase the total carbohydrate rate decrease and hence the mannanase production increase as shown in fig 2.

3.2. Effect of incubation period

On shake flask level, the production of mannanase enzyme was maximum after 168 h of incubation at 45 ºC and 200 rpm. On scale up, using the 5 litre fermentation medium on the bioreactor, the maximum production of mannanase activity was attained after 120, 114, 102 and 72 h in case of agitation speeds 200, 400, 600 and 800 rpm, respectively. The significant increase of mannanase production by batch fermentation compared to the shake flask culture could be explained by the improved oxygen transfer in the bioreactor (Blibech *et al*., 2011).

On the other hand, the specific rates diagram (fig 3) proves that the fermentation time cannot exceed 48 h while it can considered that the optimum fermentation time is 36 h. This conclusion was derived from the data as the specific substrate consumption rate remained constant after 48 h. Also, the specific growth rate and the specific production rate decreased significantly after 48 h. Also, it is safe to conclude that the mannanase production is not growth associated.

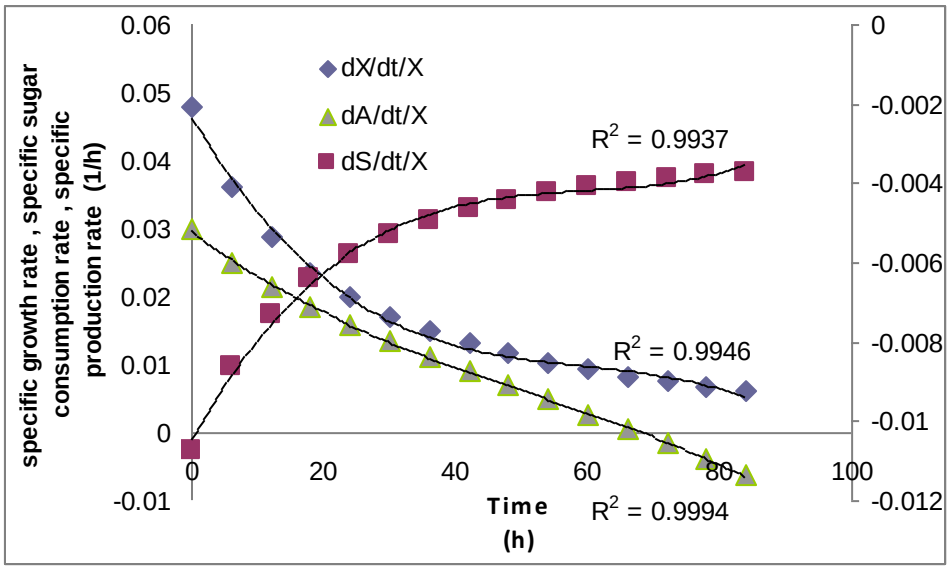

Fig. 3: Relation between time with each of specific growth rate, specific sugar consumption rate and specific production rate. . * Dissolved oxygen, (dX/dt) biomass growth rate g/L h-1, (dX/dt)/X specific growth rate 1/h, (dS/dt) sugar consumption rate g/L h-1, (dS/dt)/X specific sugar consumption rate 1/h, (dM/dt) mannanase production rate U/mL h-1, (dM/dt)/X specific mannanase production rate (U mg-1protein)/g L -1 h.

As a result of the statisitical analysis shown in fig. (4) the optimum condition for optimization of mannanase production by *B. velezensis* NRC-1 in bench scale bioreactor were agitation at 800 rpm and incubation period at 48 h.

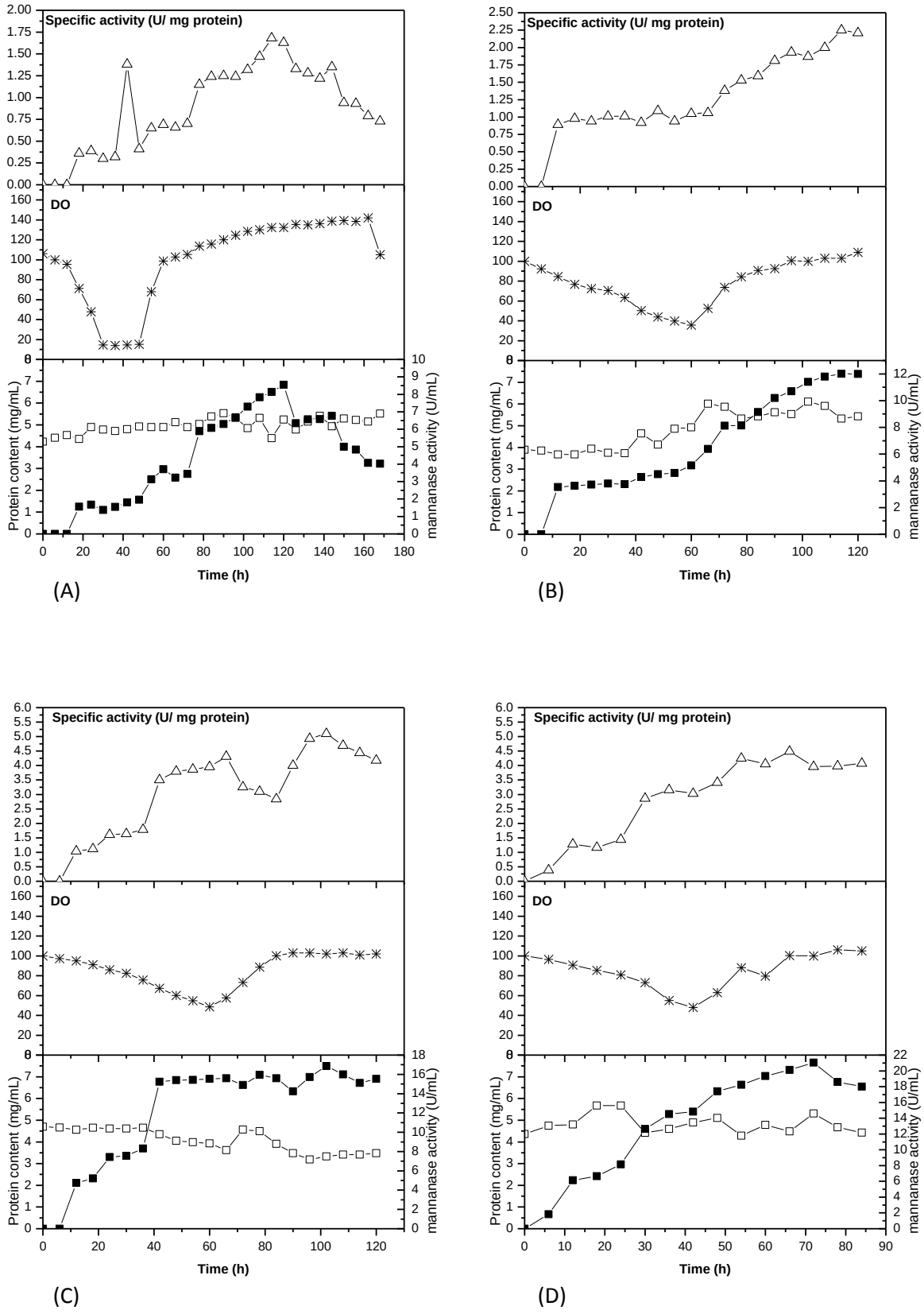

Fig. 4: β-mannanase production by B. velezensis NRC-1 at different agitation speed (A) 200 rpm, (B) 400 rpm, (C) 600 rpm and (D) 800 rpm.

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دراسة ظروف تاثير فترة الحضانة و سرعة التقليب على انتاج انزيم المننانيز (مكسر سكر المانوز) بواسطة احادى نوع بكتيرى باسيللاس فاليسينسس باستعمال المفاعل الحيوى

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تم فى هذا البحث دراسة تاثير فترة الحضانة و سرعة التقليب على انتاج انزيم المننانيز (مكسر سكر المانوز) بواسطة احادى الخلية المسمى *باسيليس فيليسينسيس* nrc-١ باستعمال المفاعل الحيوى. وقد اشارت النتائج الى ان زيادة سرعة التقليب من ٢٠٠ الى ٨٠٠ لفة بالدقيقة ادى الى زيادة انتاج الانزيم و قد ارجع هذا التغير الى زيادة كمية الاكسجين المذاب نتيجة لزيادة سرعة التقليب و الذى ادى بالتالى الى زيادة نشاط الخلايا المنتجة. وقد كانت افضل الطرق للحصول عن افضل انتاج هى ٧٢ ساعة من الاحتضان عند درجة حرارة ٤٥ مئوية. وقد اظهر الانزيم المنتج باستعمال المفاعل الحيوى نتائج افضل من تلك الناتجة عن استعمال التخمير فى القارورة المهتزة وذلك فى وقت اقل حيث تم تقليل الوقت المستخدم بطريقة القارورة المهتزة من ٧ الى ٣ ايام باستعمال المفاعل الحيوى.

الكلمات الدالة: *باسيليس فيليسينسيس* nrc-١ ، انزيم المننانيز، المفاعل الحيوى.