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Effect of Culture Conditions on Beta 1- 4 Endoglucanase Production by *Bacillus* sp.(Strain RL1)

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Abstract : : *Bacillus* sp. (Strain RL1) isolated from mountain soil was examined. This study aims to determine the effect of different carbohydrates and their concentrations {Wheat bran, Corn cob, Corn stem, Pineapple and CMC at range 0.5- 2.5 % (w/v)} as carbon sources for production of endoglucanase. Results showed that pineapple gave the highest activity 2.6993 U/ml, while the lowest activity with CMC 0.1367 U/ml. In the present investigation the endoglucanase activity was supported by 200rpm agitation rate with Corn cob and Corn stem, while 0rpm (static) revealed the best condition for endoglucanase activity using Pineapple and CMC as a sole carbon source. Within the five substrate concentrate the enzyme activity was more in 2.5 % concentration. From the overall result, it was observed that among the five carbohydrate substrate, the enzyme activity was more with Pineapple followed by Wheat bran> Corn cob>Corn stem > CMC.
Keywords: Beta 1- 4 Endoglucanase, *Bacillus* sp.(Strain RL1).

Introduction

Microorganisms in particular have been regarded as a treasure source of useful enzymes, because they multipl at extremely high rate and synthesize biologically active products which can be controlled by humans. In recent years, there has been a phenomenal increase in the use of enzymes as industrial catalysts. These enzymes offer advantage over the use of conventional chemical catalysts for numerous reasons: they exhibit high catalytic activity, a high degree of substrate specificity, can be produced in large amounts, are highly biodegradable, pose no threat to the environment and are economically viable[1]. Cellulases have got applications in many different industries such as food, brewery, wine, pulp and paper textile, detergent, feed and agriculture[2,3]. Besides, cellulases can also be used for fermentation of lignocellulosic biomass for the production of bioethanol (biofuel), which may provide the most suitable, renewable, environmentally friendly and sustainable source of energy for the future[4-6]. Three enzymes are involved in complete degradation of cellulose, i.e. endoglucanase (EC 3.2.1.4) cleaves the b-1,4-linkage in amorphous region of cellulose to yield long chain oligosaccharides; cellobiohydrolase (EC 3.2.1.91) acts on the reducing and non-reducing ends of oligosaccharides generated by the activity of endoglucanases to produce cellobiose, a dimer of glucose; and finally b-glucosidase (EC 3.2.1.21) hydrolyses cellobiose to yield glucose[7].

Cellulase production has been influenced by a number of factors including the type of strain used, the culture conditions and substrate type. The relationship between these variables has a marked effect on the ultimate production of the cellulase enzyme complex[8]. In this study, we report the effects of different substrate in promoting the production of endoglucanase enzyme by the *Bacillus* sp. RL1.

Materials and Methods

Chemicals，Media and Media Components All chemicals, media and media components used were of analytical grade obtained from Sigma Chemicals Ltd, USA and other chemical from Shanghai Chemicals Ltd, China

1. Microorganism and culture: The endoglcanase producing bacterial strain (*Bacillus* sp. RL1) was isolated from soil (Jin Yun mountain, 800 m) using serial dilution up to 10^{-9} and pour plate technique Single colonies on the plates were isolated and purified by transferring them five times onto CMC agar plates. Primary screening was performed by growing the isolate on CMC agar medium-containing-(g/L), KH_2PO_4, 2; $(NH_4)_2SO_4$,4; $MgSO_4$, 0.5 ; Peptone, 10; agar agar, 20; and distilled water, supplemented with 1% carboxy methyl cellulose (CMC) at 30°C for 24 h.

2. Identification of the isolates: The bacterial isolates obtained after the Primary screening was maintained in pure culture on CMC agar slants. All the agar slants were refrigerated at 4 °C until used. Study of colony morphology of the isolated culture was carried out followed by gram"s staining and endospore staining. Physical and Biochemical characterization of the isolated colonies was carried out using standard protocols[9]. Identification was carried out according to Burges's Manual (7th Ed.).

3. Phylogenetic analysis: The 16S rRNA gene sequence of *Bacillus* sp.RL1 was determined by direct sequencing of the purified PCR-amplified 16SrRNA gene fragment as described previously[10]. Genomic DNA was extracted by the CTAB protocol and was used as the PCR template. PCR was performed with universal bacterial primers complementary to conserved regions of the 5 and 3ends of the 16S rRNA gene, 27F (forward) (5 –AGAGTTTGATCCTGGCTCAG -3 and 1492R (reverse) (5 –GGTTACCTTGTTACGACTT -3. PCR was performed using ampliTaq gold (Applied Bio systems). The PCR products were purified with a qiaquick PCR purification kit (QIAGEN) according to the manufacturer's instruction. The purified 16S rRNA gene was sequenced directly using the ABI prism big dye terminator cycle sequencing ready reaction kit (Applied Bio systems) and an ABI prism model 377 genetic analyzer (Applied Biosystems). The obtained 16S rRNA gene sequences of isolated bacteria were compared with those from the DDBJ nucleotide sequence database using the program BLAST[11].

4. Secondary screening: A secondary screening for cellulolytic activity was conducted by using Congo red test. The bacterial isolates were grown on serial CMC agar plates containing (g/L), KH_2PO_4, 2; $(NH_4)_2SO_4$, 4; $MgSO_4$, 0.5; Peptone, 10; agar agar, 20; and distilled water, supplemented with 1% carboxy methyl cellulose (CMC) at 30°C for 24 hrs to allow the secretion of endoglucanase. Following incubation, the agar media was flooded with an aqueous solution of Congo red solution (1% w/v) and left for 15 min, the stain poured off and the plates were washed with 1M NaCl for 20 min, the solution poured off. The formation of a clear zone of hydrolysis indicated cellulose degradation[12,13].

5. Fermentation sugar: The culture was grown aerobically in a 50ml Erlenmeyer flask that contain 30ml of CMC medium containing (g/L) KH_2PO_4, 2; $(NH_4)_2SO_4$,4; $MgSO_4$, 0.5 ; Peptone, 10 and distilled water, supplemented with 1% carboxy methyl cellulose (CMC) without agar, at 35°C, pH7.0 for 72h. At the end of the incubation period, culture was centrifuged at 10'000rpmfor 10 min, endoglucanase activity was measured in the culture supernatant.

Enzyme Activity Assay

6. Endo- β-1,4-glucanase: Endo-β-1,4-glucanase activity was determined by incubation of 1ml of 1% CMC in (w/v) CMC dissolved in 0.05 M citrate buffer pH 4.8 with 1ml of appropriate concentration of enzyme at 50°C. After 30 min reaction, 1ml of dinitrosalicylic acid (DNS) was added and boiled in a water bath for 15 min to stop the reaction. The resulted samples were then cooled to room temperature and measured the absorbance at 540 nm (A540). One unit of endo-β-1-4-glucanase activity was defined as the amount of enzyme that could hydrolyze CMC and release 1 µg of glucose within 1 min reaction at 50°C[14].

7. Optimum Temperature, pH and Incubation period

Optimum Temperature, pH and Incubation period were optimized in previous investigation at 35°C, pH 7 and 72 h for enzyme production.

8. Effect of carbon sources on endoglucanase activity

Under optimized temperature, pH and incubation period. Five different carbon sources (wheat bran, corn cob, corn stem, pineapple and CMC) were tested at the different concentrations range 0.5, 1.0, 1.5, 2.0 and 2.5 % (w/v), on endoglucanase production.

9. Effect of agitation speed on endoglucanase activity

Bacillus sp. RL1 was inoculated into production medium and incubated at 35°C for 72 h in stationary phase conditions at three different conditions as follows; static, 100 rpm and 200 rpm using 50ml shaker flask. The endoglucanase activity was measured under standard enzyme assay condition.

Results:

Effect of carbon source (Wheat bran%) on endoglucanase production

Data presented in Fig.(1) shown the endoglucanase production by *Bacillus* sp. RL1 was influenced by the type and concentration of carbon source. Wheat bran was found to support maximum production at 2.5% with an enzyme activity 0.4093 U/ml. A minimum activity 0.1391U/ml was noted at 0.5 %.

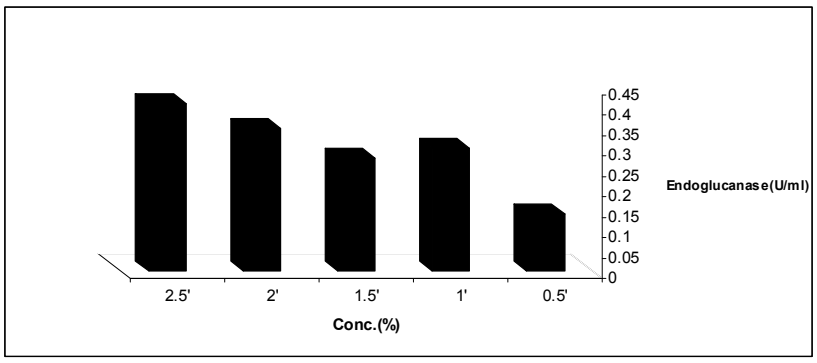

Figure 1. Effect of carbon source (Wheat bran%) on endoglucanase production (U/ml) by *Bacillus* sp. RL1, temperature37°C, pH 7 and 72 h incubation period.

Effect of carbon source (Corn cob%) on endoglucanase production

To evaluate the effect of Corn cob on endoglucanase production, the carbon source in basal medium was replaced by different concentrations of Corn cob range from 0.5- 2.5 % (w/v). Data in Fig.(2) revealed that the maximum enzyme activity was obtained with 2.5% (w/v) 0.3831U/ml, where as minimum at 0.5 %(w/v) 0.1289U/ml.

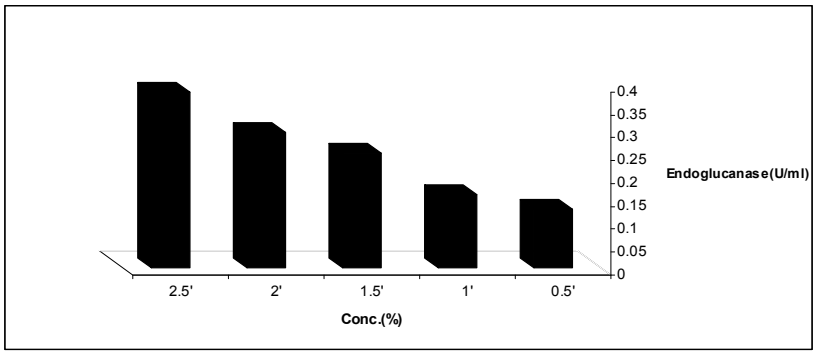

Figure 2. Effect of carbon source (Corn cob%) on endoglucanase production (U/ml) by *Bacillus* sp. RL1, temperature37°C, pH 7 and 72 h incubation period.

Effect of carbon source (Corn stem%) on endoglucanase production

In the current study it was found that 2.5 % (w/v) Corn stem revealed highest enzyme activity 0.3628U/ml and low level of enzyme was recorded at 0.5 %(w/v) 0.1045U/ml Fig.3.

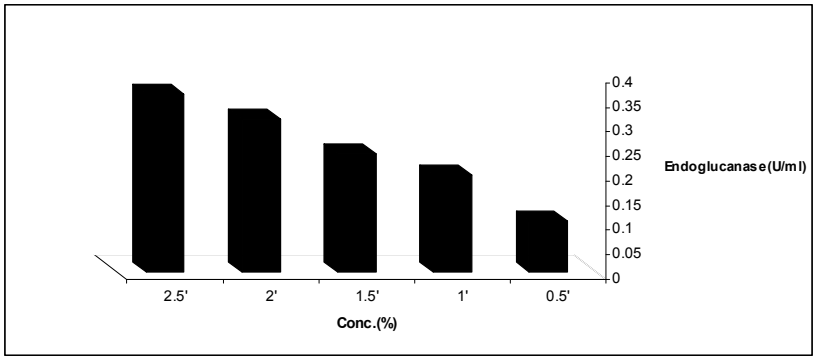

Figure 3. Effect of carbon source (Corn stem%) on endoglucanase production (U/ml) by *Bacillus* sp. RL1, temperature37°C, pH 7 and 72 h incubation period.

Effect of carbon source (Pineapple%) on endoglucanase production

Among the result presented in Fig. (4) the maximum enzyme activity was noted at 2.0%(w/v) Pineapple 2.6993U/ml, result reported lowest enzyme activity at 0.5 %(w/v) Pineapple concentration as sole carbon source in basal medium 0.3596U/ml.

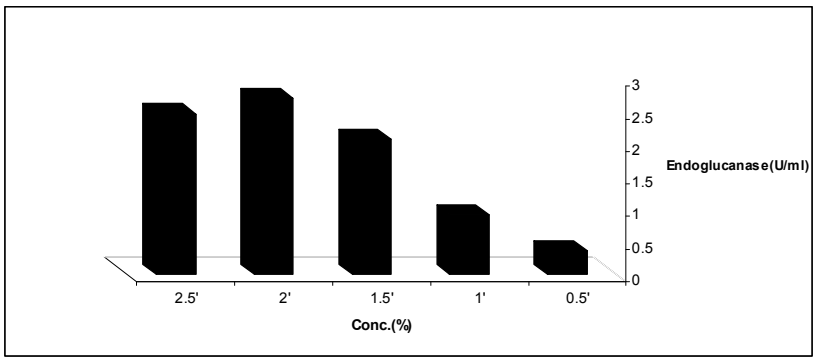

Figure 4. Effect of carbon source (Pineapple%) on endoglucanase production (U/ml) by *Bacillus* sp. RL1, temperature37°C, pH 7 and 72 h incubation period.

Effect of carbon source (CMC%) on endoglucanase production

The result presented in Fig(5) that CMC 1.0 %(w/v) was the best concentration as carbon substrate for endoglucanase production 0.1970U/ml. lowest endoglucanase activity was found at 0.5 %(w/v) 0.1367U/ml.

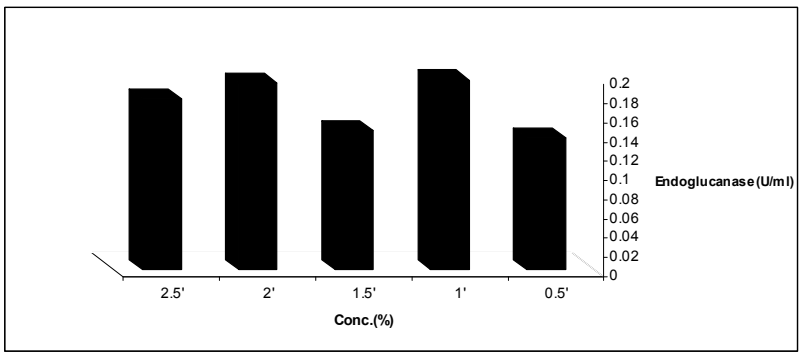

Figure 5. Effect of carbon source (CMC%) on endoglucanase production (U/ml) by *Bacillus* sp. RL1, temperature37°C, pH 7 and 72 h incubation period.

Effect of shaking (rpm) using (Wheat bran%) on endoglucanase production

With operating temperature37°C, pH 7 and 72 h incubation period, using different concentrations of Wheat bran range 0.5- 2.5% (w/v), the shaking speed was investigated by comparing the performance of the shaking rate at three rates namely 0rpm, 100rpm and 200rpm on endoglucanase production. The results were no much difference in enzyme activity produced at three shaking rates Fig. (6).

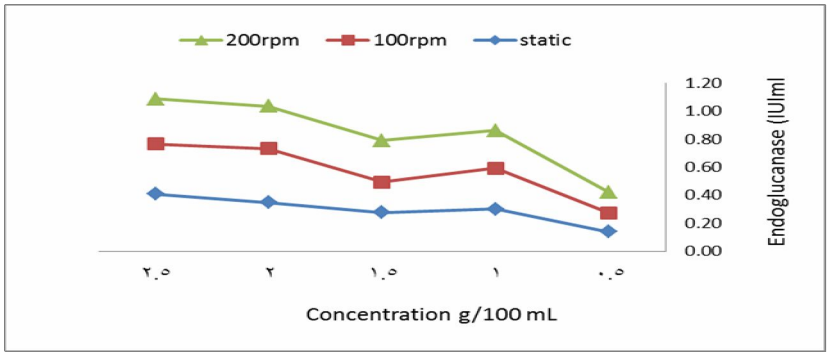

Figure 6. Effect of shaking (rpm) using (Wheat bran%) on endoglucanase production (U/ml) by *Bacillus* sp. RL1, temperature37°C, pH 7 and 72 h incubation period

Effect of shaking (rpm) using (Corn cob %) on endoglucanase production

The enzyme activity by *Bacillus* sp. RL1 at 200 rpm showed maximum activity at different Corn cob concentration(0.5, 1.0, 1.5, 2.0 and 2.5 %). Where as it was less at 100rpm with same concentrations 0.0932U/ml, 0.1448U/ml, 0.2075U/ml, 0.2835U/ml and 0.3911U/ml respectively Fig. (7).

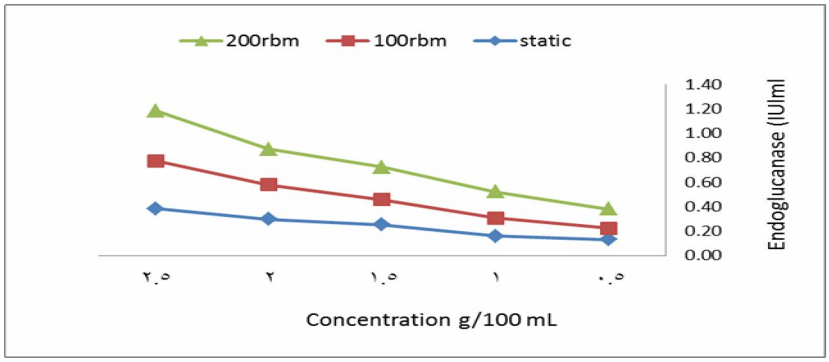

Figure 7. Effect of shaking (rpm) using (Corn cob%) on endoglucanase production (U/ml) by *Bacillus* sp. RL1, temperature 37°C, pH 7 and 72h incubation period.

Effect of shaking (rpm) using (Corn stem %) on endoglucanase production

Using different concentration of Corn stem (range 0.5, 1.0, 1.5, 2.0 and 2.5 %), as a sole carbon source at three agitation rates. Results shows a remarkable increase in enzyme production in fermentation medium under shaking condition 200rpm 0.1521U/ml, 0.2589U/ml, 0.3737U/ml, 0.4744U/ml and 0.5360U/ml, respectively, compared to 100rpm and 0rpm(static) conditions Fig.(8).

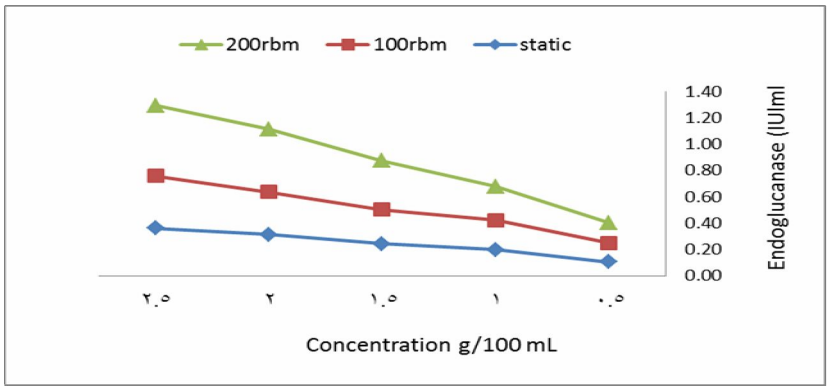

Figure 8. Effect of shaking(rpm) using (Corn stem%) on endoglucanase production (U/ml) by *Bacillus* sp. RL1, temperature37°C, pH 7 and 72h incubation period.

Effect of shaking (rpm) using (pineapple %) on endoglucanase production

With operating temperature37°C, pH 7 and 72 h incubation period, using different concentrations of Pineapple range 0.5- 2.5% (w/v),as a sole carbon source. Data presented in Fig.(9) shown that the endoglucanase production significantly increased at 0rpm conditions compared to 100 rpm and 200 rpm.

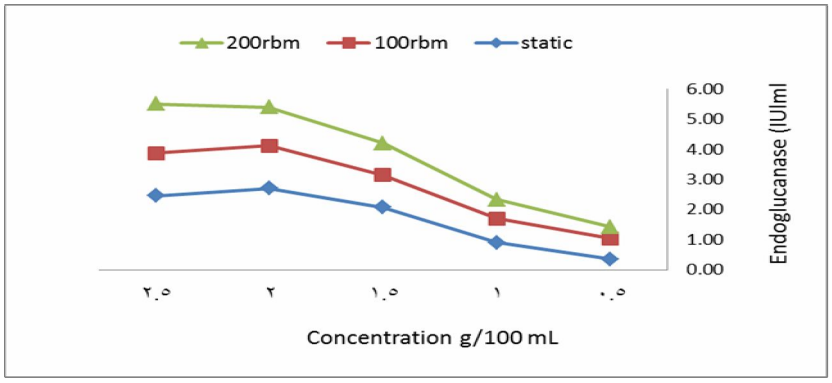

Figure 9. Effect of shaking (rpm) using (pineapple%) on endoglucanase production (U/ml) by *Bacillus* sp. RL1, temperature37°C, pH 7 and 72h incubation period.

Effect of shaking (rpm) using (CMC %) on endoglucanase production

The enzyme activity using CMC range 0.5, 1.0, 1.5, 2.0 and 2.5% (w/v), under static conditions were 0.1367U/ml, 0.1970U/ml, 0.1447U/ml, 0.1935U/ml and 0.1769U/ml respectively. However there were slight reductions in enzyme activity at 200rpm. Minimum endoglucanase activity was shown at 100rpm conditions. Fig.(10).

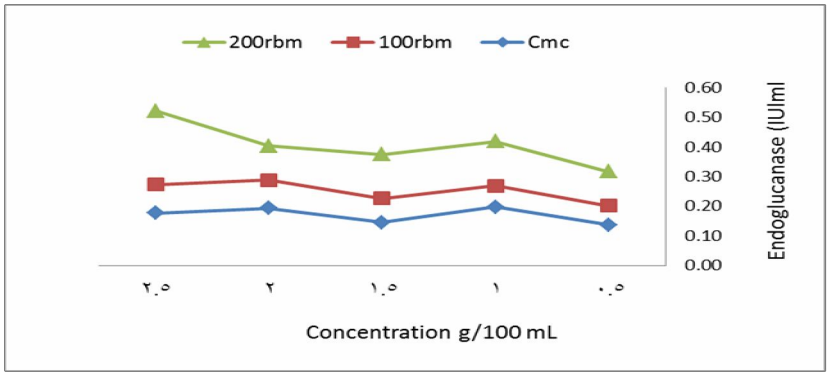

Figure 10. Effect of shaking(rpm) using (CMC%) on endoglucanase production (U/ml) by *Bacillus* sp. RL1, temperature37°C, pH 7 and 72 h incubation period.

Discussion:

In this investigation. we were studied the effect of different substrates and concentrations in promoting the activity of the cellulose degrading enzyme (endoglucanase) by *Bacillus* sp. RL1, production of the endoglucanase was determined based on the ability of this organism to grow in the presence of different substrates and concentrations (range 0.5, 1.0, 1.5, 2.0 and 2.5% (w/v) as indicated by a corresponding increase in enzyme production. Some previous studies reported that the agricultural wastes of lignocellulosics are used as a carbohydrate source to produce commercially imported products such as ethanol, glucose and single cell protein[15]. Similarly[16-21] reported some lignocellulosics as a carbon source for endoglucanase production.

In the present study various concentrations 0.5- 2.5 %(w/v) of five substrate were used as a carbon sources to find out the enzyme activity. Among the tested concentration, 2.5% showed higher enzyme activity. Some previous reports noted that the enzyme activity in *Bacillus* spp. Was maximum at 1.5%(w/v)[17], and 1.0%(w/v) as growth substrate[18].

CMC of the basal medium was replaced with alternative substrates such as Wheat bran, Corn cob, Corn stem and Pineapple. Maximum endoglucanase activity was supported by Pineapple and Wheat bran and it was quite interesting to observe that enzyme activity was enhanced on all of the crude substrates at different concentrations compared to that on pure CMC. Similar result was obtained[22] and results shows a remarkable increase in enzyme activity under shaking conditions 200rpm when used Corn cob and Corn stem as a sole carbon source. While the data revealed good endoglucanase production with static condition accompany with pineapple and CMC as sole carbon source. From the obtained data it is best supported the importance of shaking to facilitate maintenance of homogenous conditions especially with respect to temperature and other parameters[23,24].

As outlined earlier shaking plays an important role in increase the amount of dissolved oxygen and dispersion of macromolecules in the medium. It might therefore contribute to the greater growth and better enzyme production noted in current investigation. However the shearing effect induced by the higher shaking speed on the cell and enzyme activity may contribute negatively towards cell growth and enzyme stability.

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