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Nutritional and Environmental Factors Affecting Cellulase Production by Two Strains of Cellulolytic Bacilli

[1]Abou-Taleb, Khadiga A.A., [1]Mashhoor, W.A., [1]Nasr, Sohair A.,[1]Sharaf, M.S. and [2]Abdel-Azeem, Hoda H.M.

[1]Agricultural Microbiology Department, Faculty of Agriculture, Ain Shams University, Cairo, Egypt.
[2]Soil Microbiology Unit, Desert Research Center, Cairo, Egypt.

Abstract: Effect of some nutritional and environmental factors on growth and cellulase production by *Bacillus alcalophilus* S39 and *Bacillus amyloliquefaciens* $C2_3$ was investigated. Results indicated that 1% carboxymethylcellulose (CMC) and 0.7% yeast extract were most effective as the carbon and nitrogen sources respectively. Initial pH 7 and 3% inoculum size found to be optimal for growth and cellulase production. Incubation temperature at 30 and 45ºC achieved the highest activity of cellulase for *Bacillus alcalophilus* S39 and *Bacillus amyloliquefaciens* $C2_3$ respectively, and the suitable shaking rate was 150 and 200 rpm.

Key words: Nutritional & environmental factors, Cellulase production, *B. alcalophilus* S39, *B. amyloliquefaciens* $C2_3$.

INTRODUCTION

Cellulose is the most abundant biomass on the earth (Tomme *et al*., 1995). It is the primary product of photosynthesis in terrestrial environments, and the most abundant renewable bioresource produced in the biosphere (100 billion dry tons/year) (Jarvis, 2003 and Zhang & Lynd, 2004). Cellulose is commonly degraded by an enzyme called cellulase. This enzyme is produced by several microorganisms, commonly by bacteria and fungi (Bahkali, 1996; Mangelli & Forchiassin, 1999; Shin *et al*., 2000 and Immanuel *et al*., 2006).

Complete enzymatic hydrolysis of enzyme requires synergistic action of 3 types of enzymes, namely cellobiohydrolase, endoglucanase or carboxymethycellulase (CMCase) and β-glucosidases (Bhat, 2000). Cellulases are used in the textile industry for cotton softening and denim finishing; in laundry detergents for color care, cleaning, and anti-deposition; in the food industry for mashing; in the pulp and paper industries for deinking, drainage improvement, and fiber modification and they are even used for pharmaceutical applications (Kirk *et al*., 2002 and Cherry & Fidantsef, 2003).

Bacteria, which has high growth rate as compared to fungi has good potential to be used in cellulase production. However, the application of bacteria in producing cellulase is not widely used. Celluloytic property of some bacterial genera such as *Cellulomonas*, *Cellovibrio, Pseudomonas*, *Sporosphytophaga* spp. (Nakamura and Kappamura, 1982); *Bacillus and Micrococcus* (Immanuel *et al*., 2006) were also reported. Enzyme production is closely controlled in microorganisms and for improving its productivity these controls can be ameliorated. Cellulase yields appear to depend on a complex relationship involving a variety of factors like inoculum size, pH value, temperature, presence of inducers, medium additives, aeration, growth time, etc. (Immanuel *et al*., 2006).

The present work was carried out to optimize the nutritional and environmental parameters for improving cellulase production by the two cellulolytic bacterial strains.

MATERIALS AND METHODS

Microorganisms Used:

The two bacterial strains, *Bacillus alcalophilus S39* and *Bacillus amyloliquefaciens* $C2_3$ used in this study were isolated form soil and compost respectively and were distinguished as potent cellulase producers. The purified bacilli isolates were identified according to their cultural, morphological and biochemical characteristics based on Bergey's Manual of Systematic Bacteriology (Claus and Berkeley, 1986) and Biolog Automated System was used.

Corresponding Author: Hoda Hassan Abdel-Azeem, Soil Microbiology Unit, Desert Research Center, Cairo, Egypt.
E-mail: hebamashhoor@hotmail.com

Media used:

Medium (1): Nutrient agar (Difco Manual, 1984) was used for the maintenance of *Bacillus* strains.

Medium (2): Carboxymethyl cellulose medium recommended by Ray *et al*. (2007) was used for the production of cellulase by *Bacillus* sp. It has the following composition (g/l): Carboxymethylcellulose (CMC), 10; Tryptone, 2; KH_2PO_4, 4; Na_2HPO_4, 4; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.001; $FeSO_4.7H_2O$, 0.004; Agar, 15 and pH adjusted to 7.

Inoculum Preparation and Fermentation Process:

For preparation of standard inoculum, both strains were cultured in nutrient broth individually at 30 ºC for 24 h where an average viable count of 3.5 - 4.3 $\times 10^6$ cell/ml culture broth was obtained. This was used as the inoculum for the production medium.

Fermentation was carried out in 250 ml plugged Erlenmeyer flasks, each containing 50 ml sterile production medium and inoculated with 3% of standard inoculum (containing about 3.5 $\times 10^6$ and 4.3 $\times 10^6$ cell/ml for *Bacillus amyloliquefaciens* $C2_3$ and *Bacillus alcalophilus* S39, respectively). The inoculated flasks were incubated at 30 ºC and 45 ºC for *Bacillus alcalophilus* S39 and *Bacillus amyloliquefaciens* $C2_3$, respectively on rotary shaker at 150 rpm for 72h.

Preparation of Crude Enzyme:

After incubation, cultures were centrifuged at 1600 g for 15 min at 4°C and supernatants were used as source of crude enzymes. The crude enzyme solution was utilized for determination of enzyme activities (Kotchoni *et al*., 2003).

Enzyme Assays Procedures:

Carboxymethyl-cellulase(CMCase) activity:

CMCase activity was assayed using a method described by Mandels and Weber (1969). The activity was estimated using 1 % solution of carboxymethlycellulose (CMC) in 0.05 M citrate buffer (pH 4.8) as substrate. The reaction mixture contained 1 ml citrate buffer, 0.5 ml of substrate solution and 0.5 ml of suitably diluted enzyme solution. The reaction was carried out at 50°C for 30 min. The amount of reducing sugar released in the hydrolysis was measured. One unit of CMCase activity was expressed as 1 µ mol of glucose liberated per ml enzyme per minute.

Filter-paperase (FPase) Activity:

The activity of FPase was assayed according to the method explained by Mandels and Weber (1969). This method is similar to the CMCase assay method, but the substrate was Whatman No. 1 filter paper strip (1 x 6 cm) soaked in 1 ml 0.05 M sodium citrate buffer (pH 4.8). The samples were incubated with 0.5 ml enzyme solution at 50°C for 1 h, the reducing sugars liberated during growth were determined. One unit of FPase activity was determined as 1 µ mol of glucose liberated per ml enzyme per minute.

β-Glucosidase Activity:

One-tenth ml of the culture supernatant was incubated with 0.5 ml of 0.05 M acetate buffer (pH 5) containing 2.5 mg cellobiose. After incubation at 50 ºC for 10 min, the glucose released was measured by the glucose oxidase peroxidase method (Zaldívar *et al*., 2001).

Determination of Reducing Sugars:

The total amount of reducing sugars was determined using potassium ferricyanide method, as described by Park and Johnson (1949).

Carbon Sources:

The appropriate carbon source was selected by replacing the original carbon substrate of the basal medium with equivalent carbon amount of each of the tested carbon sources (Glucose, Carboxymethycellulose, Cellobiose and Cellulose).

Nitrogen Sources:

To detect the adequate nitrogen source for cellulase production by selected strains, the prescribed nitrogen source of the fermentation medium was replaced by equivalent nitrogen amount of each of the tested organic [Beef extract, Casein, Malt, Peptone, Tryptone, Urea & Yeast extract] and inorganic [KNO_3, $(NH_4)_3PO_4$, $NaNO_3$, NH_4NO_3, NH_4Cl & $(NH_4)_2SO_4$] nitrogen sources.

pH:

Seven values of pH ranged between 5.5 and 8.5 were chosen for studying their effects on cellulase enzyme to select the most suitable pH of the production medium.

Incubation Temperature:

To determine the optimum temperature for cellulase production, fermentation was carried out at various temperatures in the range of 5, 20, 25, 30, 35, 40, 45, 50 and 55 ºC.

Shaking Rate:

Erlenmeyer flasks (250 ml) containing production medium were inoculated with the selected strains and placed onto a rotary shaker at different rpm (i.e. 0, 50, 100, 150 and 200 rpm) to obtain proper aeration for maximal cellulase production.

Inoculum Size:

The inoculum size was optimized for maximal enzyme production. The fermentation medium was inoculated with 1, 2, 3, 4, 5, 6 and 7 % of standard inoculum.

Statistical Analysis:

The collected data were statistically analyzed using SPSS Computer Analysis Programs (Foster, 2001).

RESULTS AND DISCUSSION

Effect of Different Carbon Sources:

Data presented in Table (1) show that cellulase production with both two *Bacillus* strains was significantly influenced by the type of carbon source in the basal medium. Carboxymethylcellulose (CMC) was most effective as a sole carbon source for cellulase enzyme production by *Bacillus alcalophilus* S39 and *Bacillus amyloliquefaciens* $C2_3$, results in increase in enzyme activity, being 1.81 & 1.88 U/ml of CMCase activity, 0.87 & 0.86 U/ml of FPase activity and 1.31 & 1.41 U/ml of β-glucosidases, respectively. These values were followed, in descending order by cellobiose and cellulose.

These results are in agreement with those of Narasimha *et al*. (2006) and Niranjane *et al*. (2007) who found that carboxymethyl cellulose was the best carbon source followed by cellulose for cellulase production. A higher production of cellulase when CMC served as substrate may be as a result of induction of the enzyme, since cellulose is known to be a universal inducer of cellulase synthesis. Paul and Varma (1993) had reported the induction of endocellulase by CMC.

Medium containing glucose as the growth carbon source presented the minimum cellulase activity (expressed by CMCase, FPase and β-glucosidase). Muthuvelayudham and Viruthagiri (2006) obtained similar results which showed that the cellulase activity was less when glucose was used as carbon source because of inhibition.

Another experiment was carried out to study the effect of different concentrations of carboxymethylcellulose (CMC) which exhibited superiority among other tested carbon sources for *Bacillus* strains. Data in Fig. (1) clearly show that 1% carboxymethylcellulose (CMC) gave the highest activity of cellulase being 1.85 & 1.88 U/ml of CMCase; 0.87 & 0.87 U/ml of FPase and 1.35 & 1.40 U/ml of β-glucosidases by *B. alcalophilus* S39 and *B. amyloliquefaciens* $C2_3$, respectively. This is similar with previous investigations (Fukumori *et al*., 1985; Kawai *et al*., 1988 and Shikata *et al*., 1990) where the CMCase activity in *Bacillus* sp. was detected in cultures that contained 1% (w/v) CMC as the growth substrate.

Effect of Different Nitrogen Sources:

To evaluate the effect of nitrogen source on cellulase formation, the nitrogen source in the basal medium was replaced by different nitrogen sources. Data revealed that the supplementation of organic and inorganic nitrogen sources stimulated the cellulase yield and activity. Using of organic N sources responded in the positive cellulase activity more than the inorganic ones. Among the tested complex N sources, the effectiveness in supporting cellulase production and cellulolytic activity by both *Bacillus* strains significantly decreased in the following order: yeast extract > peptone > beef extract > NH_4Cl. Results recorded in Table (2) clearly show that yeast extract was the best nitrogen source for both strains giving 2.07 & 2.17 U/ml of CMCase, 0.99 & 1.01 U/ml of FPase and 2.18 & 2.55 U/ml of β-glucosidases for *Bacillus alcalophilus* S39 and *Bacillus amyloliquefaciens* $C2_3$, respectively. Data are in accordance with the results of Ray *et al*. (2007) who reported that organic nitrogen sources were found to be more suitable for optimizing cellulase production by *Bacillus subtilis* and *Bacillus circulans* than inorganic sources.

Table 1: Effect of carbon sources on the production of cellulase enzyme by the two *Bacillus* strains.

Different carbon sources	*Bacillus alcalophilus S39*				*Bacillus amyloliquefaciens* $C2_3$			
	Biomass g/100ml	Cellulase Activity (U/ml)			Biomass g/100ml	Cellulase Activity (U/ml)		
		CMCase	FPase	β- glucosidases		CMCase	FPase	β- glucosidases
Glucose	0.501^{a}	0.23^{e}	0.25^{f}	0.05^{f}	0.484^{b}	0.30^{e}	0.10^{e}	0.04^{f}
Carboxymethycellulose (CMC) (Control)	0.361^{f}	1.81^{a}	0.87^{a}	1.31^{b}	0.382^{e}	1.88^{a}	0.86^{a}	1.41^{a}
Cellobiose	0.403^{c}	0.71^{d}	0.19^{d}	0.13^{e}	0.392^{d}	1.32^{b}	0.34^{c}	0.06^{f}
Cellulose	0.157^{h}	1.00^{c}	0.41^{c}	1.27^{c}	0.295^{g}	1.43^{b}	0.51^{b}	1.24^{d}

Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan's at 5 % level.

Table 2: Effect of nitrogen sources on the production of cellulase enzyme by *Bacillus* strains.

Nitrogen sources	*Bacillus alcalophilus S39*				*Bacillus amyloliquefaciens* $C2_3$			
	Biomass g/100ml	Cellulase Activity (U/ml)			Biomass g/100ml	Cellulase Activity (U/ml)		
		CMCase	FPase	β- glucosidases		CMCase	FPase	β- glucosidases
Beef extract	0.417^{c}	2.05^{b}	0.98^{c}	2.04^{c}	0.420^{b}	1.99^{c}	0.99^{c}	2.25^{b}
Casein	0.395^{gh}	0.41^{l}	0.04^{n}	0.67^{lmn}	0.413^{d}	0.03^{n}	0.01^{o}	0.64^{mn}
Malt	0.356^{p}	1.18^{j}	0.01^{o}	0.73^{lm}	0.380^{k}	0.31^{m}	0.04^{mn}	0.68^{lmn}
Peptone	0.407^{e}	2.05^{b}	0.99^{c}	2.16^{b}	0.381^{k}	2.14^{a}	1.00^{b}	2.47^{a}
Tryptone (Control)	0.360^{o}	1.83^{f}	0.87^{i}	1.34^{h}	0.387^{i}	1.88^{ef}	0.89^{i}	1.41^{gh}
Urea	0.375^{lm}	1.50^{h}	0.47^{k}	0.70^{lmn}	0.385^{ij}	1.64^{g}	0.64^{j}	0.75^{kl}
Yeast extract	0.404^{ef}	2.07^{b}	0.99^{c}	2.18^{b}	0.417^{c}	2.17^{a}	1.01^{a}	2.55^{a}
KNO_3	0.407^{e}	1.24^{i}	0.06^{l}	1.06^{j}	0.420^{b}	0.81^{k}	0.05^{m}	0.81^{k}
$(NH_4)_3PO_4$	0.378^{l}	0.41^{l}	0.05^{m}	0.64^{n}	0.395^{gh}	1.14^{j}	0.02^{o}	0.98^{j}
$NaNO_3$	0.374^{lm}	1.88^{ef}	0.89^{h}	1.62^{e}	0.370^{lmn}	1.90^{ef}	0.90^{g}	1.10^{i}
NH_4NO_3	0.397^{g}	1.88^{ef}	0.90^{gh}	1.42^{fg}	0.371^{lmn}	1.89^{ef}	0.92^{f}	1.55^{f}
NH_4Cl	0.359^{o}	1.97^{cd}	0.96^{e}	1.77^{d}	0.361^{o}	1.93^{cde}	0.98^{c}	1.80^{d}
$(NH_4)_2SO_4$	0.424^{a}	1.91^{de}	0.92^{f}	1.49^{f}	0.417^{c}	1.91^{de}	0.97^{d}	1.55^{f}

Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan's at 5 % level.

Data illustrated in Fig. (2) obviously indicates that suitable concentration of yeast extract was found to be 0.7% which gave the highest CMCase being 2.35 & 2.30 U/ml; FPase being 1.15 & 1.19 U/ml and β-glucosidases being 3.56 & 3.49 U/ml of *B. alcalophilus* S39 and *B. amyloliquefaciens* $C2_3$, respectively.

It is notable at all experiments to state that there was no relationship between the production of cellulase enzyme and biomass yield.

Effect of initial pH:

Cellulase yield by both strains appear to depend on pH value. Results illustrated by Fig. (3) clearly show that cellulase production, expressed as enzyme activity, gradually increased as the pH values increased from 6.5 to 7.5 and reached its maximum at initial pH of 7 being 2.41 & 2.40 U/ml of CMCase, 1.19 & 1.19 U/ml of FPase and 3.55 and 3.49 U/ml of β-glucosidases by *B. alcalophilus* S39 and *B. amyloliquefaciens* $C2_3$, respectively. The cellulase activity was less in other tested pH levels, where enzyme activity was minimal at pH 5.5 and it indicates a marginal increase at pH 6.5 and 7. Further, this activity was greatly reduced to reach the lowest at pH 8.5 (where 2.06 & 2.07 U/ml of CMCase; 1.07 & 1.04 U/ml of FPase and 0.72 and 0.56 U/ml of β-glucosidases was obtained by *B. alcalophilus* S39 and *B. amyloliquefaciens* $C2_3$, respectively.

Obtained data confirmed the findings reported by Ray *et al*., (2007) who mentioned that pH 7 – 7.5 more suitable for optimization of cellulase production by *Bacillus subtilis* and *B. circulans*. Furthermore, the cellulolytic enzyme, endogluconase obtained from *Cellulomonas*, *Bacillus*, and *Micrococcus* spp. hydrolyzed substrate in the pH range of 4.0 to 9.0, with maximum activity transpiring at pH 7 (Immanuel *et al*., 2006).

Incubation Temperature:

Like pH, temperature is also an important factor that influences the cellulase yield. It is obvious from Fig. (4) that the highest cellulase activity was obtained at temperatures 30 to 45ºC for *B. alcalophilus* S39 and *B. amyloliquefaciens* $C2_3$ respectively, whereas it was less at other tested degrees for each strain.

These results are closed to those of Bakare *et al*. (2005) who found that the cellulase enzyme produced by *Pseudomonas fluorescence* was activated at 30 to 35 ºC showing the optimum temperature at 35 ºC. Ray *et al*. (2007) reported that minimum cellulase yield was observed when fermentation was carried out at 45°C, while maximum yield was obtained at 40°C by *Bacillus subtilis* and *Bacillus circulans*. Immanuel *et al*. (2006) also recorded maximum endoglucanase activity in *Cellulomonas*, *Bacillus* and *Micrococcus* sp. at 40°C and neutral pH.

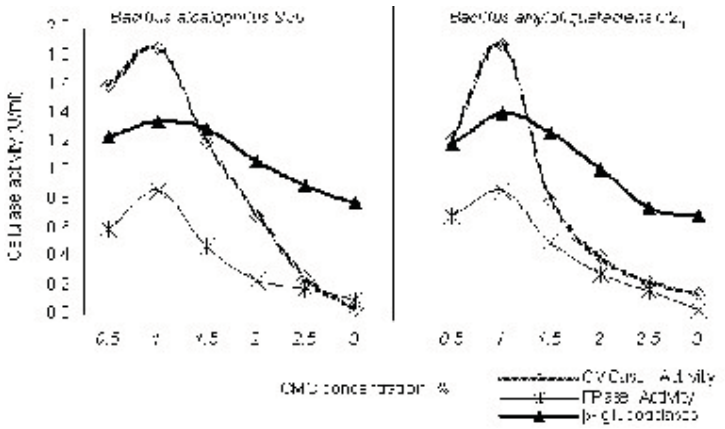

Fig. 1: Effect of different concentrations of carboxymethylcellulose (CMC) on the production of cellulase enzyme by *Bacillus* strains.

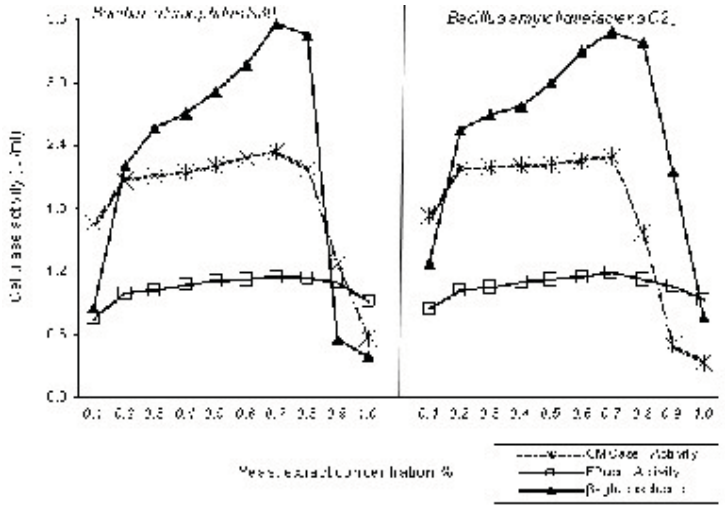

Fig. 2: Effect of different concentrations of yeast extract on the production of cellulase enzyme by *Bacillus* strains.

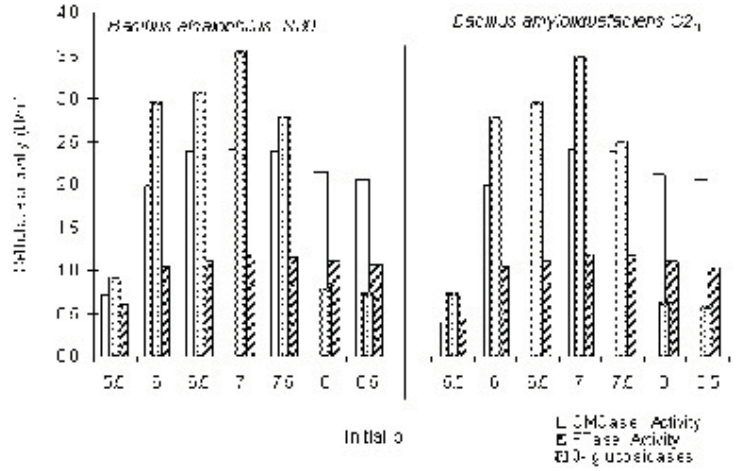

Fig. 3: Effect of initial pH on the production of cellulase enzyme by *Bacillus* strains.

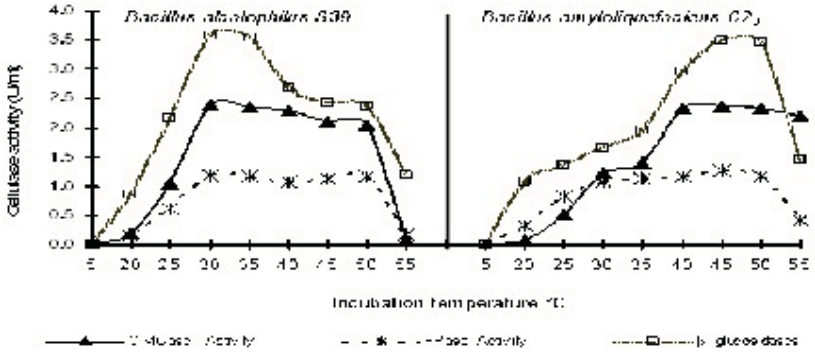

Fig. 4: Effect of incubation temperature on the production of cellulase enzyme by *Bacillus* strains.

Effect of Shaking Rate:

Concerning the effect of shaking rate on cellulase yield, it was found from the current data (Fig. 5) that the maximum activity was obtained at the range of shaking rate of 150-200 rpm for *B. alcalophilus S39* and *B. amyloliquefaciens* $C2_3$. No significantly different was noticed in enzyme activity produced at rate of 150 and 200 rpm.

Similar data was found by Bin Amwarali Khan and Husaini (2006) who noticed a remarkable increase of cellulase activity in fermentation medium under shaking condition compared to static condition. It was observed more than 2 fold higher cellulase enzyme activity in shaking condition (2.97 IU/ml) compare to non shaking condition (1.38 IU/ml) for *Bacillus amyloliquefaciens* UMAS 1002 strain. They also reported that the highest cellulase enzyme production by *Bacillus amyloliquefaciens* UMAS 1002 strain were 2.97 and 2.89 IU/ml at agitation speed of 100 and 200 rpm respectively.

Inoculum Size:

Fig. (6) shows that the inoculum size of 3.0 % achieved the highest cellulase enzyme production being 2.40 & 2.39 U/ml of CMCase; 1.20 & 1.18 U/ml of FPase and 3.61 & 3.53 U/ml of β-glucosidases by *B. alcalophilus* S39 and *B. amyloliquefaciens* $C2_3$, respectively.

These results were almost similar with findings collected by Ray *et al*. (2007) elucidated the enzyme production increased gradually up to 3% inoculum size, but decreased thereafter. The enzyme production by both strains *Bacillus subtilis* and *Bacillus circulans* in 3% inoculum size was not significantly different ($P < 0.05$) from that in 2% inoculum size.

In the present study, it could be concluded that carbon and nitrogen sources, pH value, temperature, inoculum size and aeration play a most crucial role in cellulase production by *B. alcalophilus* and *B. amyloliquefaciens*.

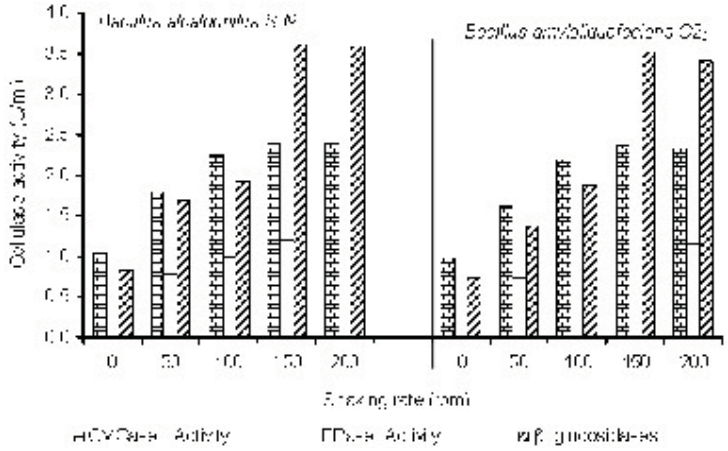

Fig. 5: Effect of shaking rate on cellulase production of by *Bacillus* strains.

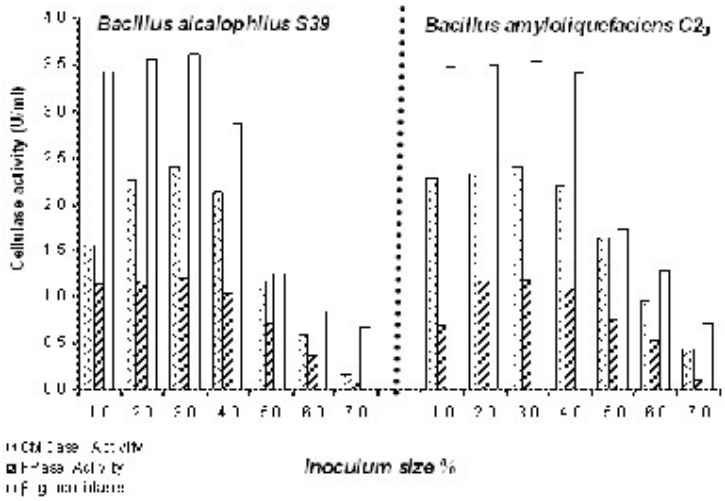

Fig. 6: Effect of inoculum size on the production of cellulase production of by *Bacillus* strains.

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