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OPTIMIZATION OF VOLUME OF FERMENTATION MEDIUM FOR THE PRODUCTION OF ALKALINE PROTEASE BY AN EMS MUTANT STRAIN OF *BACILLUS SUBTILIS* IH-72

HAMID MUKHTAR* AND IKRAM-UL-HAQ

Institute of Industrial Biotechnology,
Government College University, Lahore 54000, Pakistan.
**Corresponding author E-mail: hamidmukhtar@gcu.edu.pk*

Abstract

The study deals with the effect of volume of culture medium on the enhanced production of alkaline protease by an EMS-mutant culture of *Bacillus subtilis* in a lab scale bioreactor and shake flasks. The laboratory scale bioreactor of 7.5 L capacity was employed for the microbial cultivation experiments in addition to 250ml Erlenmeyer flasks. The parental strain of *Bacillus subtilis* was improved by inducing mutations using Ethyl methane sulphonate (EMS). After EMS-mutagenesis, 14 most potent variants showing positive mutations were isolated and screened for enhanced production of alkaline protease in shake flasks. The maximum production of the enzyme was shown by an EMS-mutant viz., EMS-8 that was 9.53U ml^{-1} and it was about 100% increase over the parental strain (4.82U ml^{-1}). The effect of the volume of fermentation medium in shake flasks and fermentor on the production of enzyme by the mutant strain was studied and it was found that optimum volume of fermentation medium in shake flasks and bioreactor was 20% and 60%, respectively. The maximum production of alkaline protease by the wild and mutant strains was 7.98 U ml^{-1} and 15.52 U ml^{-1}, respectively.

Introduction

Proteases have been classified into three categories i.e., acid, neutral and alkaline proteases on the basis of their acid-base behaviour. The acid proteases are those which have pH optima in the range of 2.0-5.0 and are mainly fungal in origin. Important examples of acid proteases are Pinguinain, Tabernamontanain, proteolytic enzymes of yeasts, *Aspergillus, Rhizopus* and *Penicillium* species (Sizer, 1961; Keay, 1971; Haq *et al.*, 2006). Proteases having pH optima in the range of 7.0 or around are called neutral proteases. They are mainly of plant origin however, some bacteria and fungi also produce neutral proteases (Haq & Mukhtar, 2005). The examples include euphorbain, pomiferain, papain, ficin, bromelain, chymopapain, and soyin etc. While proteases that have pH optima in the range of 8.0-11.0 are grouped under the category of alkaline proteases. Some of the important alkaline proteases are solanain, hurain and proteolytic enzymes of *Bacillus* and *Streptomyces* species (Hameed *et al.,* 1996; Lee *et al.,* 2002).

Alkaline proteases are produced by a great number of bacterial species (Sizer, 1964; Rao *et al.*, 1998). The bacterial proteases usually diffuse into the medium (extracellular) and can be concentrated by filtration or by precipitation. Bacteria form proteolytic enzymes when grown in protein medium however, very few bacteria produce proteases on protein- free media (Prescott & Dunn's, 2004). *Bacillus* species do possess remarkable biotechnological value due to their non-pathogenicity and ability to produce extracellular proteases in large amounts (Chandi & Subramanayam, 2002). Different species of *Bacillus* producing high titers of proteases include *B. subtilis* (Pavlova *et al.,* 1992;

Hameed *et al.,* 1996; Lee *et al.,* 2002), *B. licheniformis* (Calik *et al.,* 1998), *B. pseudofirmus* (Pedersen *et al.,* 2003), *B. cereus, B. pumilus* (Kim *et al.,* 2001), *B. stearothermophilus* (Rehman *et al.,* 1994; Peter *et al.,* 2001), *B. intermedius* (Balban *et al.,* 1993), *B. amyloliquefaciens* (George *et al.,* 1995), *B. thermoproteolyticus* (Voordouw *et al.,* 1974) and *Bacillus mycoids* (Mohamed *et al.,* 1998). Proteases are also formed by *Bacillus prodigiosus, B. proteus, B. pychyaneus, B. diphtherae* and *B. polymyxa* (Ruechle, 1923). Protease production has also been reported by alkalophilic *Streptomyces* spp., (Nakanishi *et al.,* 1974; Hiramatsu & Ouchi, 1978), *Pseudomonas* spp. (Ko *et al.,* 2002; Ravaud *et al.,* 2003), *Photorhabdus* spp. (Cabral *et al.,* 2004), *Lactobacillus* spp. (Bintsis *et al.,* 2003), *Alcaligenes faecalis* (Thangam & Rajkumar, 2002) and *Xanthmonas maltophila* (Toni *et al.,* 2002).

Proteolytic enzymes are the most important industrial enzymes, representing worldwide sales of about 60 % of total enzyme market (Woods *et al.,* 2001). They find commercial applications in a number of industries like leather industry (George *et al*., 1995), detergents (Kobayashi *et al.,* 1996) and food (Gupta *et al.,* 2002), toothpastes as antiplaque and antitartar (Hernandez & Marria, 1996), cosmetics (Ohta *et al*., 1996) and for the recovery of silver from used x-ray films (Ishikawa *et al*., 1993). The fungal proteases however, are of particular importance in the food industry (Channe *et al.,* 1998).

The volume of the fermentation medium in a shaking flask or a bioreactor and the rotating rate of a shaking bed are significant factors affecting the fermentation process. It can affect the dissolved oxygen level of the fermentation broth in a shaking flask or fermentor. The smaller the medium volume in a shaking flask, the higher is the dissolved oxygen. Also, higher rotating rate of a shaking bed is favorable for enhancing oxygen mass transfer which in turn favors the growth of the microorganism and metabolite production. The present study was undertaken to optimize the volume of fermentation medium in shake flasks and stirred fermentor to enhance the production of alkaline protease by an EMS-mutant strain of *B. subtilis* IH-72.

Materials and Methods

Bacterial strain, growth and maintenance: Stock culture of *Bacillus subtilis* IH-72 was obtained from the culture collection bank of Institute of Industrial Biotechnology, G.C. University, Lahore. The source of present strain was the soil of a tannery area. The strain was maintained by weekly transfers on to the next slants containing nutrient broth and was stored at 4°C in a cool cabinet.

EMS mutagenesis: The wild culture of *Bacillus subtilis* IH-72 was sub-cultured in nutrient broth medium overnight until a density of $3\text{-}5\times10^8$ CFU/ml was reached. The culture was then centrifuged at 6000 rpm for 10 min. The pellet was washed with sterilized saline water and resuspended in half the original volume of nutrient broth containing 0.2 M Tris (pH 7.5). EMS was then added to the bacterial suspension to the final concentration of 15 µl/ml and mixed vigorously. The tubes were then incubated in a shaking water bath at 37ºC for 30-180 min. After incubation, the culture was centrifuged and washed with sterilized saline water. The pellet was resuspended in 10 ml of nutrient broth and was allowed to grow overnight. The saturated culture was diluted up to 10^6 and plated on peptone-yeast extract-casein agar plates. The plates were incubated at 37°C for 24-48 hrs for growth of the mutagenized cells. The bacterial colonies showing bigger

zone of casein hydrolysis as compared to wild strain were picked up and transferred to the nutrient broth agar slants.

Inoculum development and culture medium: Bacterial cells from newly prepared 48 hrs old slants were inoculated into a flask containing preculture medium consisting of (g/L) peptone from meat, 5.0 and yeast extract, 3.0 (pH 7.2). The flask was incubated at 37°C on a rotary shaker (Gallenkamp, UK) at 200 rev min^{-1} for 24 hrs. This inoculum was used to carry out fermentation experiments.

The basal medium used for the production of alkaline proteases by *Bacillus subtilis* IH-72 consisted of (% w/v) soybean meal, 2.0; polypeptone, 1.0; yeast extract, 0.2; glucose, 1.5; KH_2PO_4, 0.1; NH_4SO_4, 0.1 and Na_2CO_3, 0.5 (pH 8.5).

Fermentation experiments: The shake flask studies were carried out in 250 ml Erlenmeyer flasks. The flasks were charged with the fermentation medium, cotton wool plugged and sterilized at 121°C (15 lbs/in^2 pressure) for 15 min. After cooling, the sterilized flasks were inoculated with freshly prepared bacterial inoculum and incubated at 37°C on a rotary shaker (Gallenkamp, UK) at 200 rpm for 48 hrs.

The fermentation experiments were also carried out in a laboratory scale 7.5 L batch bioreactor (New Bruinswick Scientific, USA) with a working volume of 5.0 L. The bioreactor was equipped with monitors, which were used to measure and control foam, temperature, pH, stirring rate and dissolved oxygen. The vessel of the bioreactor was equipped with a four-blade turbine.

A peristaltic pump was used to control the foam by automatic addition of an antifoam silicon agent. For tests with automatic pH control, a system operating with an ingold sterilizable electrode and automatic addition of oxalic acid / KOH solutions through peristaltic pump was used. Controls were performed at different levels of different parameters.

Kinetic and statistical analysis: Kinetic parameters for batch fermentation experiments were determined according to the methods as described by Pirt (1975) and Lawford & Rouseau (1993). The following parameters of kinetics were studied:

i. Maximum specific growth rate $(\mu)_{max}$ per hour - The value of $(\mu)_{max}$ was calculated from plot of lnx vs. time of fermentation.

ii. Product yield coefficient ($Y_{p/x}$) U/ml/mg - The value of $Y_{p/x}$ was determined by the equation:

$$Y_{p/x} = dp/dx$$

iii. Specific product yield coefficient (q_p) U/ml/h - The value of q_p was determined by the equation:

$$Y_{p/x} \,.\, (\mu)_{max}$$

The experimental data was statistically analyzed by the method of Snedecor & Cochrane (1980). Duncan's multiple range test was applied under one way ANOVA. Significance has been presented in the form of probability ($p \leq 0.05$) values.

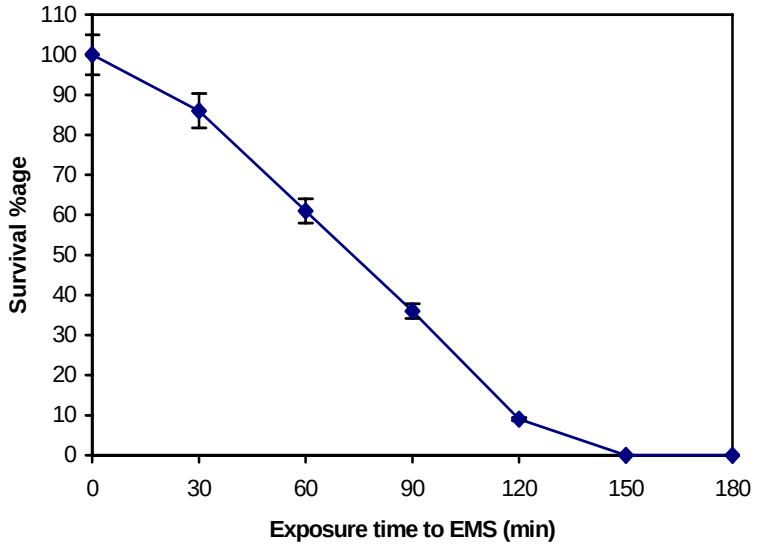

Fig. 1. Survival curve of *Bacillus subtilis* IH-72 treated with EMS @ 15µl/ml.

Assay of protease: The method of McDonald & Chen (1965) was used for the assay of protease. Casein (1.0%) was used as substrate which was incubated with the enzyme sample. After incubation, the unhydrolysed casein was precipitated by 5% TCA. The blue colour produced with Folin and Ciocalteau reagent was measured at 700nm for optical density. One unit of protease activity is defined as the amount of enzyme required to produce an increase of 0.1 in optical density at 700 nm under defined conditions.

Results

The wild strain of *Bacillus subtilis* IH-72 was treated with EMS for different time intervals ranging from 30-180 min at a concentration of 15 µl/ml. The survival curve of the wild strain was drawn and absolute death was observed at a treatment of 150 min (Fig. 1). The mutants so produced were isolated on the basis of larger zone of casein hydrolysis as compared to wild on the isolation plates containing peptone-yeast extract-casein agar. The isolated mutants were examined for the production of alkaline protease in shake flasks (Table 1). The results of the screening showed that mutant EMS-8 gave maximum production of the enzyme (9.53 ± 0.03 U/ml) and was selected for further studies. The mutant was assigned the code *Bacillus subtilis* IH-72^{EMS-8}.

The effect of different volumes of the fermentation medium in shake flasks on the production of alkaline protease by *Bacillus subtilis* IH-72 was investigated. The volume of the fermentation medium varied from 25-125 ml per 250 ml Erlenmeyer flask. The enzyme production was markedly influenced by the volume of the fermentation medium contained in the shaken flasks (Fig. 2). The biosynthesis of enzyme followed by growth of the microorganism was increased with increase in the volume of the medium from 25 to 50 ml where it was maximum {5.78 U/ml (W) & 7.76 U/ml (M)}. As the volume of fermentation medium was increased above 50 ml, both the growth of the organism and production of the enzyme decreased gradually and reached minimum when the volume of culture medium was 125 ml.

Table 1. Screening of EMS treated mutant strains of *Bacillus subtilis* IH-72 for enhanced proteolytic activity in shake flasks*.

Mutant code	Protease activity (U/ml)	H/C ratio
EMS-1	6.83±0.03^{e}	3.62±0.02^{f}
EMS-2	6.41±0.03^{f}	3.45±0.03^{g}
EMS-3	7.22±0.03^{c}	3.87±0.02^{d}
EMS-4	5.83±0.03^{j}	3.04±0.05^{k}
EMS-5	7.6±0.02^{b}	4.00±0.02^{b}
EMS-6	6.24±0.05^{g}	3. 33±0.04^{h}
EMS-7	6.05±0.05^{h}	3.25±0.04^{i}
EMS-8	**9.53±0.03^{a}**	**4.32±0.01^{a}**
EMS-9	7.61±0.04^{b}	3.93±0.06^{c}
EMS-10	5.9±0.02^{i}	3.14±0.02^{j}
EMS-11	6.41±0.02^{f}	3.42±0.02^{g}
EMS-12	5.34±0.05^{k}	2.76±0.02^{l}
EMS-13	7.12±0.03^{d}	3.82±0.01^{e}
EMS-14	6.23±0.05^{g}	3.35±0.05^{h}
Wild strain	4.82±0.03	2.14±0.02
LSD:	0.0484	0.0499

Each value is a mean of three parallel replicates. ± Indicates the standard deviation from the mean value. Values differ significantly at p≤0.05.
*Fermentation medium as in materials & methods portion, pH 8.0, incubation temperature 35°C, incubation time 48 hrs.

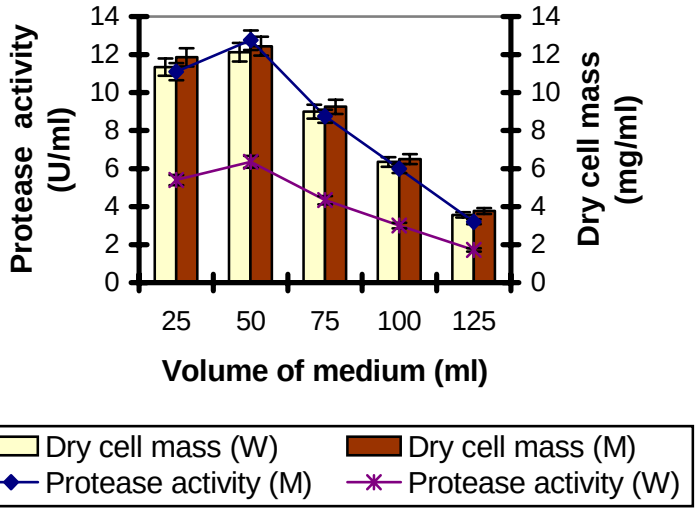

Fig 2. Effect of volume of fermentation medium on the production of alkaline protease by *Bacillus subtilis* IH-72 and its mutant derivative in shake flasks*.

Each value is an average of thr ee parallel replicates. Y bars indicate the standard error of mean value.
*Initial pH 8.5, incubation temperature 37ºC, fermentation period (W) 48 hrs, fermentation period (M) 42 hrs.

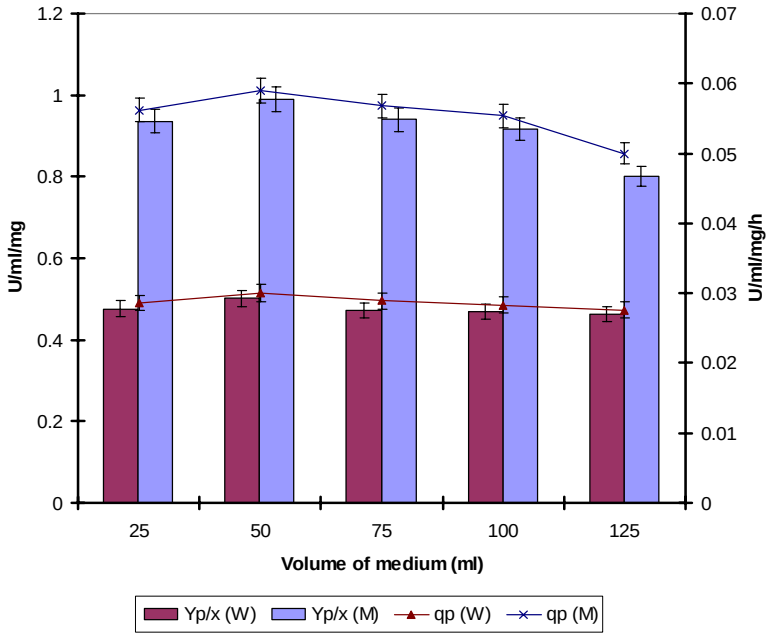

Fig. 3. Kinetic evaluation of different volumes of culture medium for the production of alkaline protease by wild and mutant strains of *Bacillus subtilis* IH-72 in shake flasks.

Kinetic parameters

$Y_{p/x}$ = Product yield coefficient (U/ml/mg)

q_p = Specific rate of enzyme formation (U/ml/mg/h)

The experimental data was subjected to kinetic analysis and the values of $Y_{p/x}$ and q_p were determined. It was observed that the optimum values of the said kinetic constants were obtained when the volume of fermentation medium was kept at 50 ml/250 ml flask (Fig. 3). The other values of fermentation volume were not significant and thus not selected. So 50 ml of fermentation medium contained in 250 ml conical flask was found optimum for alkaline protease production.

The data of Figs. 4 & 5 shows the effect of different volumes of the basal medium on the production of biomass and alkaline protease by *Bacillus subtilis* IH-72 in a stirred bioreactor, respectively. The volume of the fermentation medium varied from 40-75% of the total volume of fermentor vessel and maximum growth {12.3 mg/ml (W) & 12.58 mg/ml (M)} and production of alkaline protease {7.4 U/ml (W) & 11.12 U/ml (M)} was achieved when the fermentation batch was operated at a basal volume of 65%. The data also shows that as the volume of fermentation medium was increased above 65%, the production of the enzyme started to decrease gradually. The growth of the organism and production of enzyme at lower volumes of basal medium (below 65%) were also insignificant. The dissolved oxygen content was also monitored during the fermentation process (data not shown) to observe the effect of increasing volume of fermentation medium on the oxygen supply to the microorganism. The maximum dissolved oxygen (40%) was observed at a working volume of 65%, while all other levels of volume had lesser amount of dissolved oxygen as compared to the optimum.

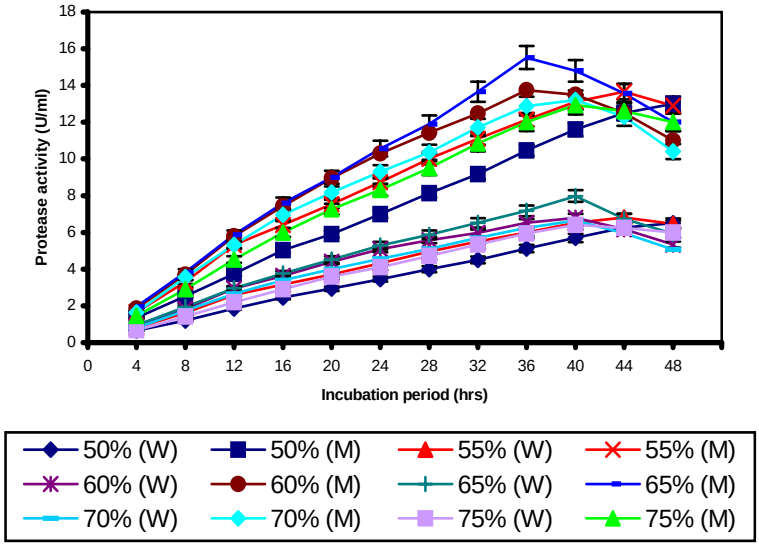

Fig. 4. Effect of volume of fermentation medium on the production of alkaline protease by *Bacillus subtilis* IH-72 and its mutant derivative in stirred fermentor*.

Each value is an average of three replicates. Y error bars indicate the standard error among the values.
*Medium pH 8.5, incubation temperature 37°C, agitation 225 rpm, aeration rate 0.6 vvm, inoculum size, 3% (v/v).

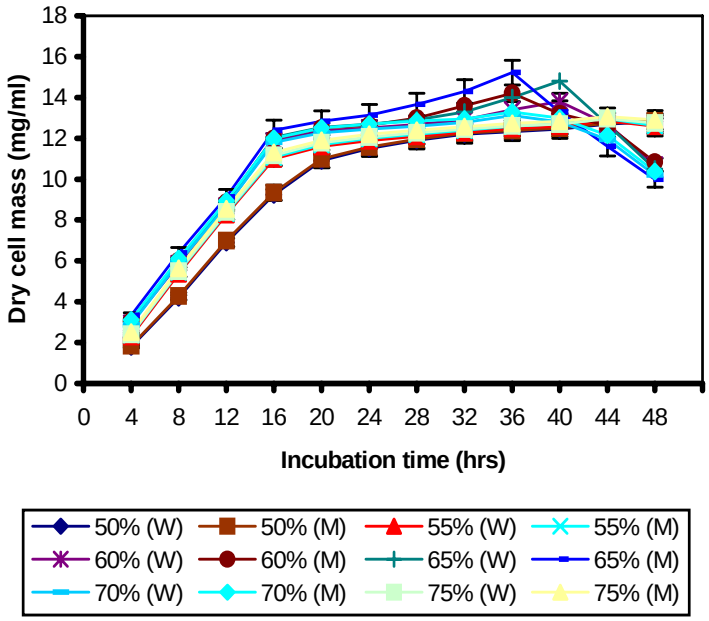

Fig. 5. Effect of different volume of fermentation medium on the production of biomass by *Bacillus subtilis* IH-72 and its mutant derivative in stirred fermentor*.

Each value is an average of three replicates. Y error bars indicate the standard error among the values.
*Medium pH 8.5, incubation temperature 37°C, agitation 225 rpm, aeration rate 0.6 vvm, inoculum size, 3% (v/v).

Table 2. Kinetic evaluation of different volumes of fermentation medium for the production of alkaline protease by wild and mutant strains of *Bacillus subtilis* IH-72 in a fermentor.

Working volume (%)	Kinetic parameters*							
	$Y_{p/x}$ (W)	$Y_{p/x}$ (M)	q_p (W)	q_p (M)	Q_p (W)	Q_p (M)	Q_x (W)	Q_x (M)
50	0.458	0.845	0.0321	0.0591	0.14	0.27	0.27	0.274
55	0.517	0.963	0.0362	0.0674	0.16	0.31	0.27	0.292
60	0.511	1.023	0.0358	0.0716	0.17	0.37	0.33	0.369
65	**0.569**	**1.088**	**0.0398**	**0.0762**	**0.19**	**0.37**	**0.371**	**0.372**
70	0.519	0.969	0.0363	0.0678	0.17	0.33	0.367	0.368
75	0.505	0.943	0.0354	0.0660	0.16	0.32	0.29	0.297

***Kinetic parameters**

$Y_{p/x}$ = Product yield coefficient (U/ml/mg); Q_p = Volumetric rate of enzyme production (U/ml/h); Q_x = Volumetric rate of biomass formation (mg/ml/h); q_p = Specific rate of enzyme formation (U/ml/mg/h).

The kinetic values $Y_{p/x}$ and q_p were also calculated and it was found that the significant values of above parameters were obtained when the volume of the fermentation medium was kept at 65% and varied significantly ($p<0.05$) than the values at other volumes of basal medium. So 65% volume of fermentation medium in the fermentor vessel was optimized for the production of alkaline protease by both the wild and EMS-mutant *Bacillus subtilis* IH-72 in the fermentor.

Discussion

The volume of the fermentation medium is of great significance in the production of enzymes from *Bacillus* species using submerged fermentation. It affects the growth of the organism and production of enzymes through influencing agitation and aeration of the culture medium (Jin *et al*., 2004). The maximum protease production during shake flask studies was obtained when 20 % volume (50ml/250ml flask) of the medium was used. At this volume of the culture medium, growth of bacterium was optimal due to a greater oxygen supply, resulting in the maximum enzyme production. Further increase in the volume of medium resulted in the decreased production of alkaline protease by the organism. It might be due to the improper agitation and inadequate aeration (oxygen supply) resulting from higher volumes. Similar kinds of findings have also been reported by Viitanen *et al.,* (2003) and Ekwealor & Obeta (2005) who have optimized 20 and 25 % volume of the medium for the production of lysine and single cell proteins by *Bacillus megaterium* and *E. coli* respectively. However, Ali *et al*., (2001) optimized 10% volume of the fermentation medium in 250 ml Erlenmeyer flasks for the fungal fermentations.

Volume of the fermentation medium in the vessel of bioreactor is also one of the significant factors in the fermentor studies as it is known to directly affect the agitation and aeration of the culture and indirectly microbial growth and enzyme production (Roza *et al.*, 2002). The maximum production of alkaline protease {7.4 U/ml (W) & 14.92 U/ml (M)} was achieved when the fermentation batch was operated at a basal volume of 65%. The degree of agitation and aeration was greater in small volumes of the fermentation medium as compared to the higher volumes that was one factor for the differences in enzyme productions. Under improper agitation and aeration, desired homogeneity in the culture medium was also not achieved which was another factor making a hindrance in the growth of microorganism and enzyme biosynthesis. Moriel *et al.,* (2005) have also optimized the working volume of fermentor vessel as 60-65% for astaxanthin production.

Conclusion

Volume of the basal medium is one of the significant factors to be optimized for the production of enzymes and other metabolites during submerged fermentation. The optimization of volume of fermentation medium has resulted in an overall increase of growth and protease production by *Bacillus subtilis*, so it has a strong economic bearing when large scale fermentation experiments are carried out.

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