OPTIMIZATION OF ALKALINE PROTEASE PRODUCTION BY *STREPTOMYCES AMBOFACIENS* IN FREE AND IMMOBILIZED FORM

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ABSTRACT

Optimization of alkaline protease production by *Streptomyces ambofaciens* NRRL 2420 in free and immobilized form was investigated using submerged fermentation technique. The optimum conditions for maximum alkaline protease production 342 unit mL^{-1} were 30°C at pH 8.5 and incubation time 96 h in free cell cultures using starch 20 g L^{-1} as carbon source and yeast extract 5 g L^{-1} as nitrogen source. The incubation time for the best yield of 344 unit mL^{-1} was reduced to 72 h under the optimized fermentation conditions by immobilized cells adsorbed on synthetic cotton fibers. Data obtained during 5 reusable cycles showed higher levels of enzyme in shorter time duration. Immobilization of *Streptomyces ambofaciens* NRRL 2420 on synthetic cotton fiber permit repeated reuse of the cells under the optimized fermentation conditions.

Keywords: *Streptomyces ambofaciens*, Alkaline Protease, Immobilization, Adsorption

1. INTRODUCTION

Protease enzyme catalyses the hydrolysis of proteins into peptides and amino acids and considered as one of the most useful enzyme groups. Alkaline proteases have found a wide application in several industrial processes, such as laundry detergents, protein recovery or solubilization as well as in the production of protein hydrolysates, meat tenderizations, in bating of hides and skins in leather industries, for the film industry and in waste processing (Gupta *et al.*, 2002; Amit *et al.*, 2011). It constitute 60-65% of the global industrial market (Banerjee *et al.*, 1999). While most of the studies on actinomycetes have focused on antibiotic production, only few reports are on their enzymatic potential (Chaphalkar and Deys, 1998; Schippers *et al.*, 2002; Tsuchiya, 1997). The possibility of using *Streptomyces* for protease production has been investigated because of

capacity to secrete the proteins extracellular in the media which is generally regarded as safe with food and drug administration (GRAS). *Streptomyces* that produce protease include *S. clavuligerus*, *S. griseus*, *S. rimosus*, *S. thermoviolaceus* and *S. thermovulgaris* (James *et al.*, 1991). They produce variety of extracellular proteases that have been related to aerial mycelium formation and sporulation (Kim and Lee, 1995). Studies on *Streptomyces clavuligerus* cultures for protease production showed that the amount of enzyme produced varies greatly with the culture media used (Porto *et al.*, 1996). Proteases from *Streptomyces* origin offer an advantage as the mycelium can be easily removed by filtration (Phadatare *et al.*, 1993). By using immobilized cells, the protease can be produced in a shorter reaction time. Further, the rate of protease production can be improved over that of submerged batch fermentation. The long-term stability of the immobilized cells during

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the course of fermentation and the easy separation of enzyme also make them promising candidates for commercial exploitation. The most widely used immobilization method is entrapment, but the adsorption method has two advantages namely its simplicity and better physiological conditions (Pramod and Lingappa, 2008). Batch (Ozawa *et al.*, 1992) and repeated batch (Ozawa *et al.*, 1996) fermentation processes were also demonstrated using urethane foam as an immobilization carrier. As the composition of culture media strongly influences enzyme production (Giarrhizzo *et al.*, 2007). The objective of this study was to optimize cultural condition for the biosynthesis of extracellular alkaline protease from *Streptomyces ambofaciens* in free and immobilized form to obtain higher yield of this enzyme.

2. MATERIALS AND METHODS

2.1. Microorganism and Culture Conditions

Streptomyces ambofaciens NRRL 2420 used in this study was obtained from Agricultural Research Service (ARS) Culture Collection (NRRL), USDA, Peoria, IL. The microorganism was maintained at 30°C on ISP-2 agar slants made up of malt extract (1.0%), yeast extract (0.4%), glucose (0.4%), agar-agar (2.0%) (Pridham *et al.*, 2002).

2.1.1. Inoculum Preparation

The inoculum was prepared by transferring a loop full of slant culture into 50 mL of sterile broth containing (g/L): Glucose 4, yeast extract 4 and malt extract 10 $MgSO_4, 7H_2O$ 0.5. pH was adjusted to 7. The inoculum was incubated at 30°C on a rotary shaker (200 rpm) for 48 h.

2.1.2. Fermentation Protocol

The initial fermentation medium composed of (g/L): Glucose 10, yeast extract 5, $K_2 HPO_4$ 0.5 and $MgSO_4, 7H_2O$ 0.5 and pH 7. Media were autoclaved at 121°C for 20 min. Inoculation of 2 mL vegetative growth was carried out in 250 mL Erlenmeyer flasks containing 50 mL of the fermentation medium followed by the estimation of protease activity.

2.2. Analytical Methods

Alkaline protease activity of culture filtrate was determined by calorimetric method (Hagihara, 1958) using casein as the substrate. 0.5 mL enzyme solution was added to 3.0 mL casein solution (0.6% w/v casein solution prepared in 20mM Borax-NaOH buffer, (pH 10) and allowed to react for 10 min at 37°C. The reaction was terminated by the addition of 3.2 mL stopper solution TCA mixture (0.11M TCA, 0.22M sodium acetate, 0.33M acetic acid). The reaction mixture was then kept for 10 min the absorbance was measured at 280 nm and related to the protease activity.

2.3. Optimization of Different Growth Conditions

The effect of pH on alkaline protease production was determined by adjusting pH of the basal media ranging from 7-11. Optimum inoculum size was studied by incubating the medium with inoculum ranging from 1-5 percent. Influence of temperature on protease production was determined by incubating the culture media at temperatures ranging from 30-40°C. After the incubation, the culture was filtered to separate the clear supernatant. The supernatant was analyzed for protease activity.

2.4. Effect of Different Carbon Sources on Alkaline Protease Production

Glucose was substituted with (0.3% w/v) starch, sucrose, mannose, maltose and lactose. All carbon sources were autoclaved separately. After sterilization of media, sugars were added separately and the microorganism was inoculated and incubated for 144 h at 30°C and 200 rpm.

2.5. Immobilization and Cultivation of *Streptomyces ambofaciens* NRRL 2420

Synthetic cotton fiber as adsorbent was cut into small parts about 0.5 g/flask and washed several times with distilled water before use. Three ml of 48 hours vegetative growth were inoculated into each Erlenmeyer flasks (250 mL). Each flask contained 50 mL of sterilized medium. Flasks were inoculated and incubated in an incubator shaker at 200 rpm and 30°C.

2.6. Production of Alkaline Protease by Repeated Batch Process

The reusability of *Streptomyces ambofaciens* cells immobilized on synthetic cotton fiber was examined. After attaining the maximum production of alkaline protease, the spent medium was replaced with fresh production medium (50 mL) and the process was repeated for several batches until the enzyme titers were not detected. All experiments were carried out in duplicate and average values were reported.

2.7. Statistical Analysis

The data in experiments were analysed using Analysis of Variance (ANOVA) from Minitab 16 software with 95% confidence interval to investigate if there is a statistically significant difference in the data recorded since it is necessary to determine if real difference exists between the means of the values.

3. RESULTS

3.1. Culture Conditions for Alkaline Protease Production

3.1.1. Effect of Initial pH of Fermentation Medium

Different pH, 6.5, 7, 7.5, 8, 8.5, 9, 10 and 11 were adjusted in the cultural media for fermentation process. The production increased gradually by increasing the initial pH till reaching the optimum pH 8.5 at which highest protease activity was achieved (**Fig. 1**). Further increase in pH reduced enzyme production significantly. The effect of initial pH was significant on alkaline protease production ($p < 0.05$) indicate that the factor level observations means are different.

The individual 95% confidence intervals provide one-sample t intervals that estimate the mean response for each group level.

One-way ANOVA: Protease activity (U/mL) versus pH

Source	DF	SS	MS	F	P
pH	7	9767.044	1395.292	3630.03	0.000
Error	8	3.075	0.384		
Total	15	9770.119			

S = 0.6200   R-Sq = 99.97%   R-Sq(adj) = 99.94%

DF: Degree of Freedom; SS: Sum Square; MS: Mean Square; F: F-test; P: Probability test

Individual 95% CIs for Mean Based on Pooled StDev

Level	N	Mean	StDev	
6.5	2	54.500	0.707	(*
7.0	2	69.850	0.212	*
7.5	2	87.550	0.636	*
8.0	2	92.500	0.707	*
8.5	2	112.500	0.707	*
9.0	2	97.500	0.707	*
10.0	2	61.750	0.354	(*
11.0	2	31.500	0.707	(*

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                 ------------------+---------+---------+---------+-
                                  50        75       100      125
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Pooled StDev = 0.620; N: The number of observations for the level; StDev: Standard Deviation
The * indicates the sample mean value
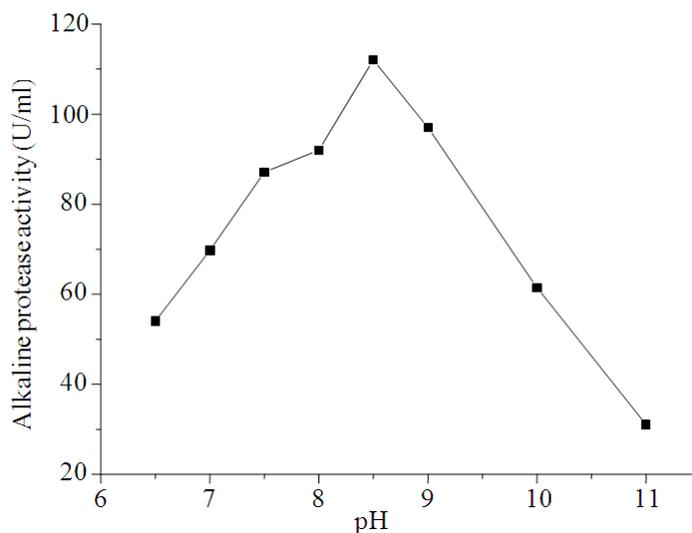
Fig. 1. Effect of different pH on alkaline protease activity

The interval plot shows that pH 6.5; 8.5 and 11 groups are definitely different in means as their intervals do not overlap. It can be concluded that the difference between the means are significant at confidence intervals 95%.

3.1.2. Effect of Different Incubation Time

Protease activity was detected only after 24 h of incubation period recorded a weak activity of (16.6 U mL^{-1}). Production was increased gradually by time till reached its maximum value of (112 U mL^{-1}) at the fourth day of the fermentation process followed by gradual decrease in activity by the 6th day reaching 31 U mL^{-1}. At the 7th day no activity was detected (**Fig. 2**). The effect of incubation time was significant on alkaline protease production since ($p < 0.05$).

One-way ANOVA: Protease activity (U/mL) versus incubation time

Source	DF	SS	MS	F	P
Incubation time	6	30588	5098	28.09	0.000
Error	14	2541	181		
Total	20	33128			

S = 13.47   R-Sq = 92.33%   R-Sq (adj) = 89.04%

Individual 95% CIs for mean based on pooled StDev

Level	N	Mean	StDev	----+---------+---------+---------+-----
24	3	8.10	1.01	(---*---)
48	3	16.20	1.06	(---*---)
72	3	81.00	1.00	(---*---)
96	3	111.67	1.53	(---*---)
120	3	57.00	35.51	(---*---)
144	3	31.00	1.00	(---*---)
168	3	1.00	1.73	(---*---)
				----+---------+---------+---------+-----
				0 40 80 120

Pooled StDev = 13.47
N: The number of observations for the level; StDev: Standard deviation
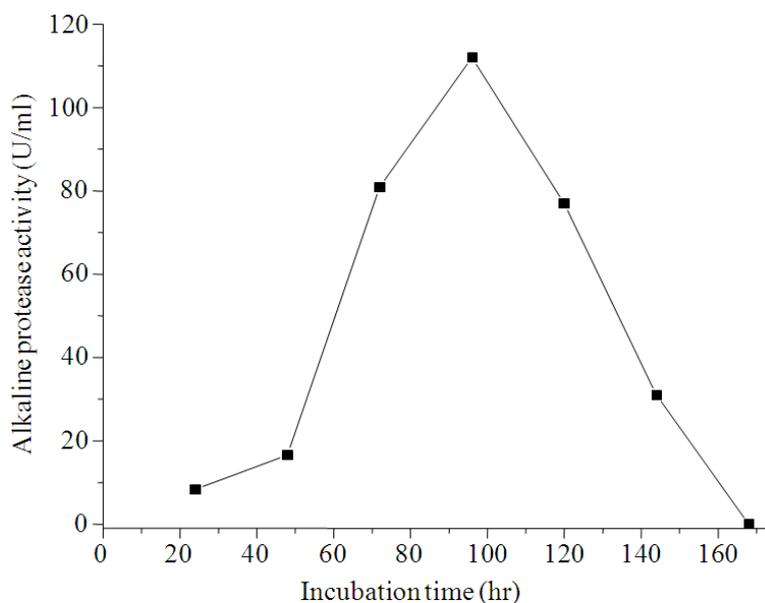

Fig. 2. Effect of different incubation time on alkaline protease activity

The interval plot shows that incubation time 24; 72 and 96 groups are definitely different in means. Their intervals do not overlap. It can be concluded that the difference between the means are significant at confidence intervals 95%.

3.1.3. Effect of Different Medium Volume

The highest productivity was attained at medium volume of 50 mL/flask yielded 112 U mL^{-1} furthermore, a decrease in the enzyme level have been observed with the increase in medium volume (**Fig. 3**), then the yield decreased by increasing the volume of the fermentation medium till reaching 100 mL/flask only 24.39 U mL^{-1} was detected. The effect of medium volume was significant on alkaline protease production ($p<0.05$).

The interval plot shows that medium volume 25; 50; 75 and 100 groups are definitely different in means. Their intervals do not overlap. It can be concluded that the difference between the means are significant at confidence intervals 95%.
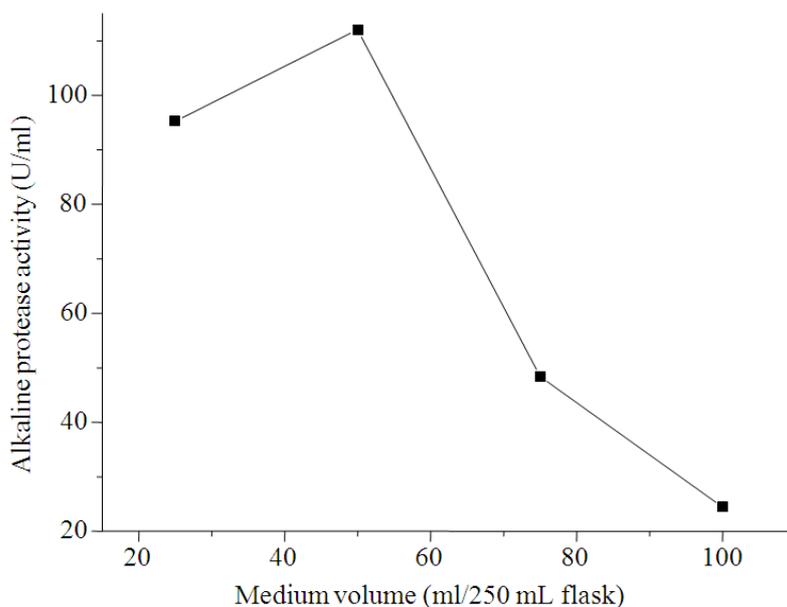
One-way ANOVA: Protease activity(u/ml) versus medium volume(ml/flask)

Source	DF	SS	MS	F	P
medium volume(ml/flask)	3	9938.32	3312.77	2140.85	0.000
Error	4	6.19	1.55		
Total	7	9944.51			

S = 1.244   R-Sq = 99.94%   R-Sq(adj) = 99.89%

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	-+---------+---------+---------+--------
25	2	96.12	1.24	(*)
50	2	113.00	1.41	(*)
75	2	49.18	1.16	(*)
100	2	25.20	1.14	(*)

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                                 -+---------+---------+---------+--------
                                 25       50        75        100
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Pooled StDev = 1.24

Fig. 3. Effect of different medium volume on alkaline protease activity

3.1.4. Effect of Different Temperature

Temperature is one of the critical parameters that has to be optimized. The optimum temperature for alkaline protease production was found to be 30ºC. Considerable enzyme production was observed between 30-35 (**Fig. 4**). The effect of temperature was significant on enzyme production ($p < 0.05$).
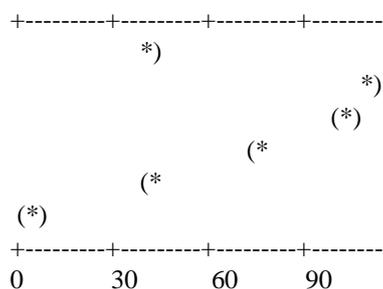
One-way ANOVA: Protease activity (u/mL) versus temperature

Source	DF	SS	MS	F	P
Temperature	5	18825.61	3765.12	3924.15	0.000
Error	6	5.76	0.96		
Total	11	18831.36			

S = 0.9795   R-Sq = 99.97%   R-Sq(adj) = 99.94%

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	
25	2	39.515	0.728	*)
30	2	111.500	0.707	*)
35	2	107.900	1.273	(*)
40	2	67.860	1.216	(*
45	2	34.900	1.273	(*
50	2	2.060	0.085	(*)

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          +---------+---------+---------+---------
          0        30        60        90
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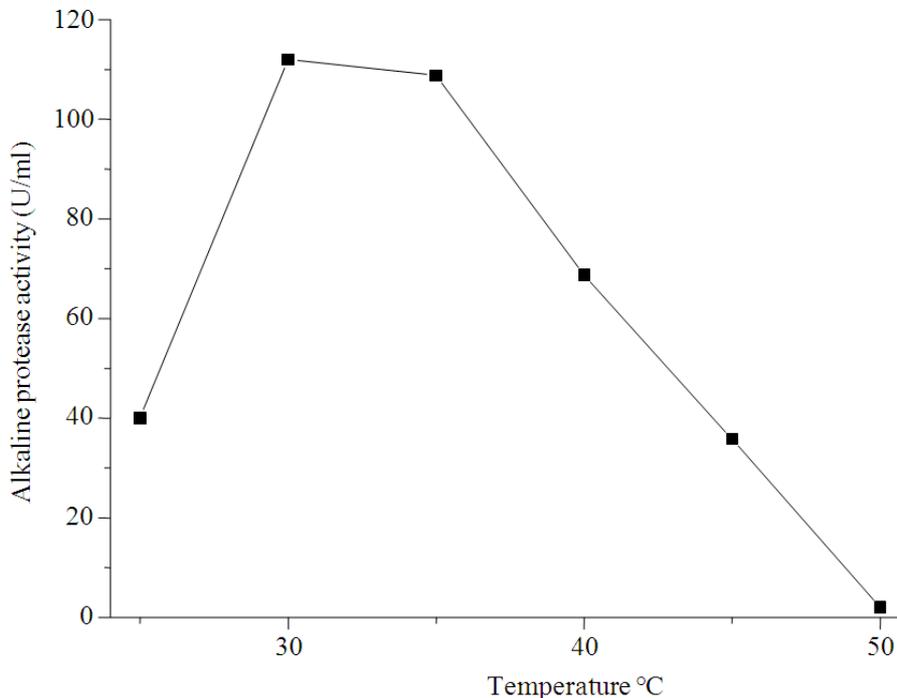
Pooled StDev = 0.980

Fig. 4. Effect of different temperature on alkaline protease activity

3.1.5. Effect of Different Carbon Sources

The basal production medium for protease production possesses glucose as carbon source. When glucose was replaced by various sugars, starch, sucrose, fructose, maltose, lactose and galactose. Starch was found to be the most effective carbon source for protease production compared with the others (**Fig. 5**). The effect of carbon sources was significant on the production (p<0.05). When starch concentrations increased from 5 to 20 g L^{-1}, an approximately linear relationship between alkaline protease yielding and the starch concentration was observed until maximum protease production were attained (342.18 unit mL^{-1}) at 20 g L^{-1} starch as listed in **Table 1**. However, increasing starch concentrations above 20 g L^{-1} was concomitant with decreased alkaline protease yield.

The interval plot shows that carbon sources lactose and maltose are definitely equal in means hence the difference between the means are not significant for production at confidence intervals 95%.
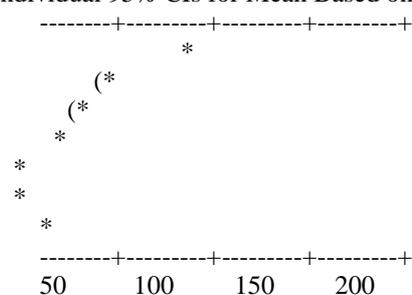
One-way ANOVA: Protease activity versus carbon sources

Source	DF	SS	MS	F	P
C.S	6	39530.49	6588.42	26707.33	0.000
Error	7	1.73	0.25		
Total	13	39532.22			

S = 0.4967   R-Sq = 100.00%   R-Sq (adj) = 99.99%

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                                    Individual 95% CIs for Mean Based on Pooled StDev
Level   N      Mean      StDev       --------+---------+---------+---------+-
1       2      193.625   0.530                              *
2       2      132.940   0.085                  (*
3       2      112.500   0.707                (*
4       2      100.640   0.509              *
5       2      69.588    0.583       *
6       2      69.588    0.583       *
7       2      10.990    0.014         *
                                     --------+---------+---------+---------+-
                                         50       100       150       200
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Pooled StDev = 0.497

1: Starch; 2: Sucrose; 3: Glucose; 4: Fructose; 5: Lactose; 6: Maltose; 7: Galactose
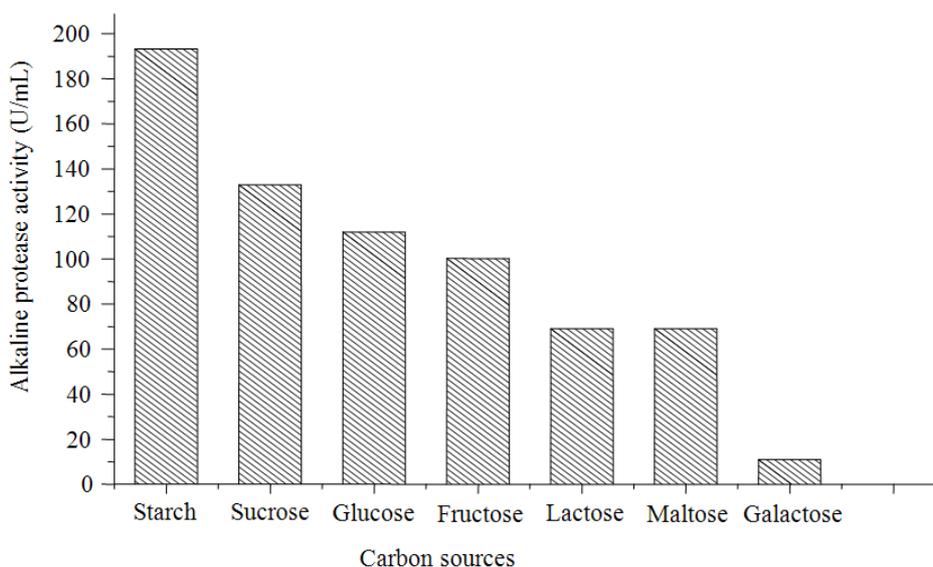

Fig. 5. Effect of different carbon sources on alkaline protease activity

One-way ANOVA: Protease activity (U/mL) versus Starch concentration (g/L)

Source	DF	SS	MS	F	P
Starch concentration (g/	5	139846.1	27969.2	34458.99	0.000
Error	6	4.9	0.8		
Total	11	139850.9			

S = 0.9009   R-Sq = 100.00%   R-Sq(adj) = 99.99%

Individual 95% CIs for Mean Based on Pooled StDev

Level	N	Mean	StDev	
5	2	35.900	0.141	*)
10	2	193.500	0.707	*
15	2	259.130	1.230	*)
20	2	342.590	0.580	*
25	2	331.000	1.414	*)
30	2	325.500	0.707	(*

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------+---------+---------+---------+---
     80       160       240       320
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Pooled StDev = 0.901

The interval plot shows that starch concentration at 5; 10; 15 are 20 are definitely different in means hence the difference between the means are significant for production at confidence intervals 95%.

3.1.6. Effect of Different Nitrogen Sources

Yeast extract was replaced by various organic and inorganic nitrogen sources in the production medium. Among the inorganic nitrogen sources, ammonium sulphate was found to be better among them for the production of the enzymes. However, among the organic nitrogen sources tested, yeast extract exhibited a prominent effect on the yield of protease, while peptone gave lower yield of protease. Casein cause a decrease in enzyme yield (**Table 2**). It was observed that the effect of nitrogen sources was significant on alkaline protease production ($p < 0.05$).

One-way ANOVA: Protease activity (U/mL) versus nitrogen sources

Source	DF	SS	MS	F	P
Different nitrogen source	6	108026.7	18004.5	148499.10	0.000
Error	7	0.8	0.1		
Total	13	108027.6			

S = 0.3482   R-Sq = 100.00%   R-Sq(adj) = 100.00%

Individual 95% CIs for Mean Based on Pooled StDev

Level	N	Mean	StDev	
Ammonium nitrate	2	93.770	0.325	*
Ammonium sulphate	2	140.900	0.141	*
Casein	2	75.970	0.042	*
Peptone	2	92.845	0.219	*
Potassium nitrate	2	95.620	0.537	*
Sodium nitrate	2	85.845	0.219	*
Yeast extract	2	342.590	0.580	*

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        140       210       280       350
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Pooled StDev = 0.348

The highest yield was noticed with 5 g L^{-1} yeast extract when used as the sole nitrogen source in the presence of 20 g L^{-1} starch instead of glucose. At higher concentrations, the enzyme secretion was repressed (**Fig. 6**). It was observed that the effect yeast extract was significant on alkaline protease production ($p<0.05$). However, 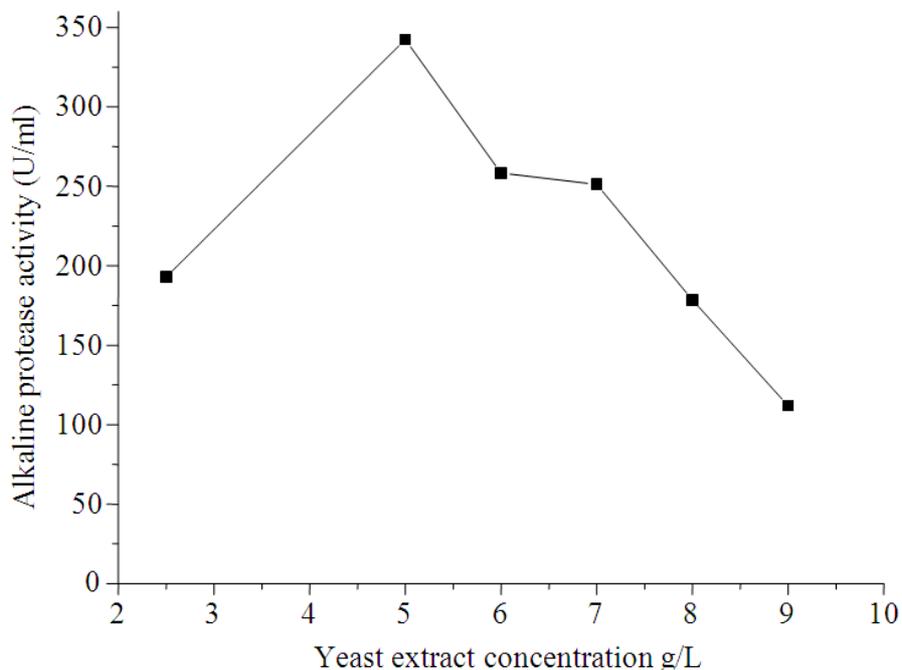there is no report on the nitrogen repression of alkaline protease from actinomycetes. After optimization of the cultural composition of the production medium for the biosynthesis of extracellular alkaline protease by *Streptomyces ambofaciens* NRRL 2420, the results indicated that the cultural filtrate possessed about 342 U mL^{-1}. This medium composition was therefore used in all succeeding work.

One-way ANOVA: Protease activity (U/mL) versus yeast extract concentration (g/L)

Source	DF	SS	MS	F	P
Yeast extract concentrate	5	62770.13	12554.03	25159.63	0.000
Error	6	2.99	0.50		
Total	11	62773.12			

S = 0.7064   R-Sq = 100.00%   R-Sq(adj) = 99.99%

Individual 95% CIs for Mean Based on Pooled StDev

Level	N	Mean	StDev	
2.5	2	193.500	0.707	
5.0	2	342.590	0.580	
6.0	2	258.630	0.523	
7.0	2	251.630	0.523	
8.0	2	179.255	1.054	
9.0	2	112.500	0.707	

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Individual 95% CIs for Mean Based on Pooled StDev
            -+---------+---------+---------+--------
                                    *
                                                *
                                            *
                       *
                               *
             *
            -+---------+---------+---------+--------
           120       180       240       300
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Fig. 6. Effect of different yeast extract concentration on alkaline protease production
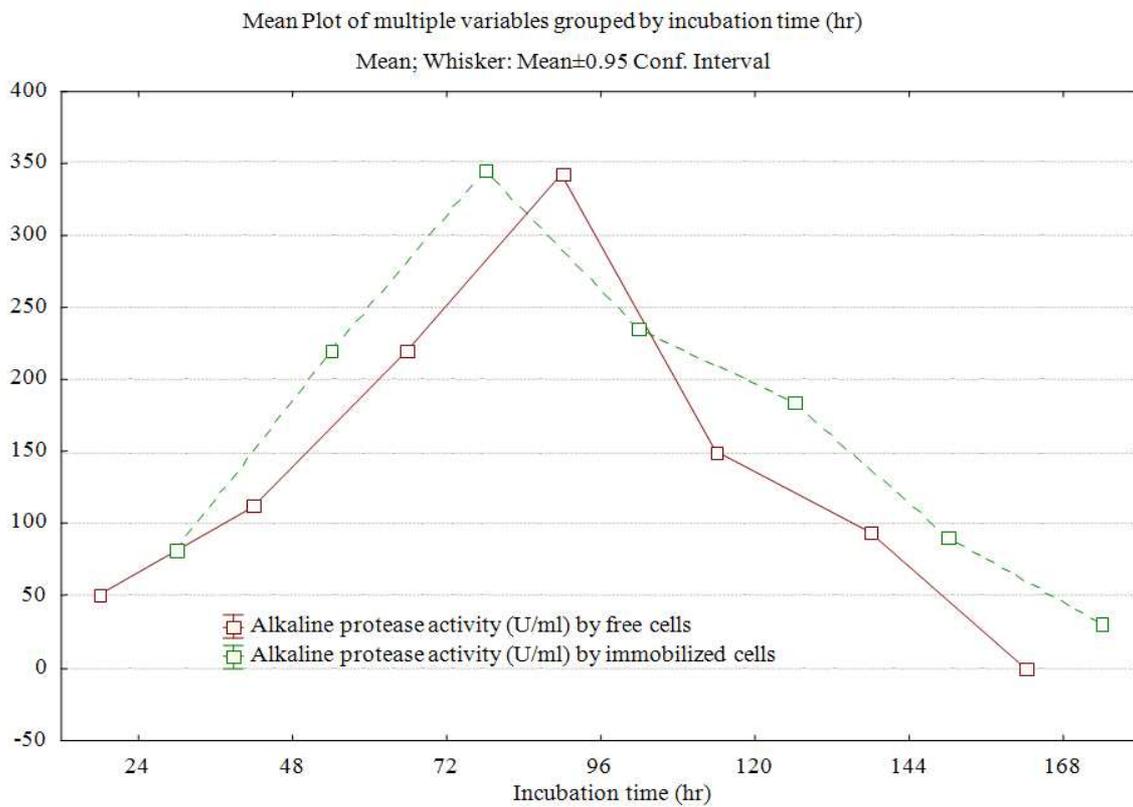
Fig. 7. Comparison between the production of alkaline protease *by Streptomyces ambofaciens* NRRL 2420 in free and immobilized form. Empty objects depict the mean and whiskers 0.95 the confidence interval
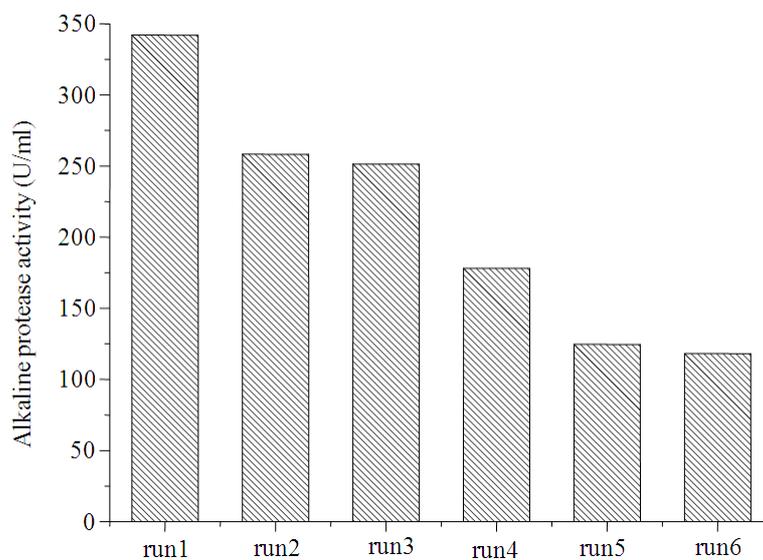
Fig. 8. Repeated batch cultivation using immobilized cells of *Streptomyces ambofaciens* NRRL 2420

Table 1. Effect of different starch concentration on alkaline protease production

Starch concentration (g/L)	Alkaline protease activity U/mL
5	35.80
10	193.00
15	258.26
20	342.18
25	330.00
30	325.00

Table 2. Effect of different nitrogen sources

Different nitrogen sources	Alkaline protease activity (U/mL)
Yeast extract	342.18
Ammonium sulphate	140.80
Potassium nitrate	95.24
Ammonium nitrate	93.54
Peptone	92.69
Sodium nitrate	85.69
Casein	75.94

The interval plot shows that yeast extract concentration at 6 g L^{-1} and 7 g L^{-1} are definitely equal in means hence the difference between the means are not significant for production at confidence intervals 95%. Immobilization of *Streptomyces ambofaciens* NRRL 2420 by adsorption on synthetic cotton fiber was investigated under the optimized conditions for alkaline protease production by free cells. The analysis of enzyme production was carried out at 24 h intervals. In case of free cells, enzyme production reached a maximum activity of 342 μ mL^{-1} at 96 h of incubation. On further incubation, the enzyme activity gradually decreased. However, the cells immobilized on synthetic cotton fiber showed a significant increase in the production from the beginning of the cultivation process. Maximum enzyme titer (344 μ mL^{-1}) was observed at 72 h of incubation. STATISTICA sigma software (Version 8.0, StatSoft Inc., USA) was used to plot the graph of **Fig. 7**. The enzyme production by immobilized cells reached approximately 220 μ mL^{-1} at 48 h, which could only be obtained after 72 h with free cells. From the results obtained, it is evident that the production of alkaline protease by immobilized cells is higher than that of free cells. The possible reuse of *Streptomyces ambofaciens* NRRL 2420 onto synthetic cotton fiber for alkaline protease production in the semi-continuous mode was carried out by replacement of the cultural medium by a fresh one under aseptic condition each 3 days over a period of 15 days (**Fig. 8**). The highest enzyme activity of 342.18 μ mL^{-1} and 258.26 μ mL^{-1} were recorded in the first and second runs. Further

replacement of the medium had no positive effect on enzyme productivity which remained between 251.26-124.66 μ mL^{-1} throughout the third and fourth cycles followed by a decrease in the enzyme activity until the fifth run reached 118.1 μ mL^{-1}.

4. DISCUSSION

Media pH plays an important role in the activity of enzymes. The present protease exhibited great activity in the pH 8.5. It has been reported that a pH of 9.0 to be optimum for alkaline protease production by *Streptomyces pulvereceus* MTCC 8374 (Jayasree *et al*., 2009). These results were synonymous to that have been investigated by (Nadeem *et al*., 2008). The results of the effect of incubation time confirmed the observation of which reported that an increase of protease production by *Streptomyces cyanens* and *S. rimosus* during log phase (Petinate *et al*., 1999; Yang and Wang, 1999). This indicates that high level of protease production is observed during active biomass production. Based on the results of the previous studies, high concentrations of oxygen had a stimulatory effect on microbial growth and enzyme production (Yegneswaran and Gray, 1991). Temperature is an important environmental factor for growth and enzyme production. The obtained result was in agreement with previous reports for protease production from streptomycetes (Chaphalkar and Deys, 1998; Kim and Lee, 1995; Yeoman and Edwards, 1994). Increasing of the alkaline protease was reported by several workers using different sugars such as fructose (Sen and Satyanarayana, 1993) and lactose (Malathi and Chakraborty, 1991). Reports concluded that yeast extract excited the biosynthesis of protease from streptomyces species (Laluce and Molinari, 1977; Yang and Lee, 2001). The use of inorganic nitrogen sources supported a moderate production of protease which matching the results obtained by some reports (Sinha and Satyanarayana, 1991; Chaphalkar and Deys, 1998). Although some carbon and nitrogen sources showed stimulatory effect on the microbial growth, they had an inhibitory effect on the biosynthesis of primary metabolites because of organic acid accumulation, oxygen depletion and glucose catabolic repression. Same phenomena were also found in protease production in *Streptomyces rimosus* (Yang and Lee, 2001). Cell immobilization is one of the common techniques for increasing the overall cell concentration and productivity. The separation of products from immobilized cells is easier compared to suspended cell systems (Beshay, 2003). The enzyme production profiles

reveal a clear difference between the activities of free and immobilized cells in fermentation processes. The immobilized-cell system leads to higher enzyme activity than that of the free-cell system during the same period of fermentation. This may be due to the depletion of essential nutrients required for the growth of the microorganism free cells. Furthermore, the long term stability and reusability of the matrix also add to the specific advantages. The higher production in case of immobilized cells could be due to the minimal growth of cells in the matrix and also due to enzyme activities being retained at higher levels than that of the free cells (Kar and Ray, 2008). A 32% increase in pectinase production by *Streptomyces* sp. RCK-SC was reported after employing a polyurethane foam as an inert support matrix (Kuhad *et al.*, 2004). Similarly, a 1.5 fold increase in polygalactouronase production by *Bacillus* sp. MG-cp-2 using the polyurethane foam as support matrix (Kapoor *et al.*, 2000).

5. CONCLUSION

This is the first report on alkaline protease production by (*Streptomyces ambofaciens* NRRL 2420). The optimized conditions of the fermentation media can be implemented in large scale for production of alkaline proteases.

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