Available online at www.pelagiaresearchlibrary.com

Pelagia Research Library

Pelagia Research Library

Advances in Applied Science Research, 2014, 5(3):315-324

Pelagia Research Library

ISSN: 0976-8610
CODEN (USA): AASRFC

Fed batch and batch submerged fermentation for alkaline protease production from mixed consortium of *Pseudomonas putida* and *Staphylococcus aureus*

S. Radha*, T. Hari Prasad, N. Syamala Devi and S. Mamata

Department of Biotechnology, S. V. Engg. Collge, Sainath Nagar, Tirupati, A.P., India

---

ABSTRACT

First report on the production of protease through fed batch cultivation using co-culture of Pseudomonas putida and Staphylococcus aureus from a crude carbon source, banana peel. Batch production by using synthetic medium, impact of some of the process parameters on cell growth and alkaline protease activity was investigated and the optimized values were found to be inoculum size of 5 % (v/v), period of cultivation as 72 hr, incubation temperature of 40^0C, and initial pH of production medium as 8.0. Synthetic medium was improved with the addition of dried and ground banana peel as the main carbon source and was economized with the replacement of its nitrogen source of synthetic medium, yeast extract, by soybean meal. Further the effective concentrations were found to be 2% (w/v) banana peel and 1% (w/v) soybean meal with the alkaline protease activity of 1443.67±5.6 U/ml through batch fermentation. Mathematical models were proposed for the effect of temperature, incubation time, and pH of cultivation medium. The suitability of these proposed models was checked with ANOVA. Higher concentrations of banana peel (> 2% (w/v)) and soybean meal (> 1% (w/v)) were found to inhibitory to alkaline protease activity. This problem was overcome by shifting batch to fed batch fermentation with constant supply of standard medium of banana peel (2% (w/v)) and soybean meal (4% (w/v)) at 70hr of operation. Specific growth rate was continuously decreased due to dilution of cultivation medium while the concentration of bacterial cell mass and alkaline protease were found to increase and finally reached a constant value. Fructose which was the main carbon component of banana peel was utilized by cells and kept its value as low as 1.2 g/l. Enzyme activity in fed batch fermentation was 1.81 fold - increase than batch fermentation.

Key words: Mixed consortium, batch, fed batch, alkaline protease, banana peel, soybean meal

---

INTRODUCTION

Proteases are hydrolytic enzymes with E.C.3.4. 21- 25 that catalyze the cleavage of peptide bonds of other proteins [1-3]. These constitute the largest product segment in industrial enzymes market with carbohydrases and lipases, as they have been using in leather, detergents, textile, food and feed and pharmaceutical industries [4-6]. Based upon pH of cultivation medium, these can be classified as acidic, neutral and alkaline proteases [7]. Currently, these enzymes are produced through batch, fed batch and continuous modes while fed batch and continuous cultivations with supply of carbon and nitrogen sources are generally superior to batch processing for longer incubation periods [8]. Particularly fed batch is preferred to batch when higher concentrations of nutrients may inhibit cell productivity thereby affect yield of the desired product [9-11].

Research on proteases is ongoing process and the production of these enzymes locally will save the much needed foreign exchange [12]. Especially, enormous published data are available on protease production from *Bacillus* sp [6]. Additionally, it's synthesis from *Pseudomonas* and *Staphylococcus* sp. have been reported either in submerged or solid state fermentation [13] while much has not been reported from the mixed combination of *Pseudomonas putida* and *Staphylococcus aureus*. Therefore the present investigation was performed with following objectives:

- To check the potentiality of co-consortium of *Pseudomonas putida* and *Staphylococcus aureus* for alkaline protease production in batch submerged fermentation
- To optimize pH, incubation time, concentration of inoculum, and incubation temperature
- To economize batch synthesis of alkaline protease by using fruit waste as main carbon source
- To formulate a mathematical model for the computation of alkaline protease activity
- To enhance the alkaline protease activity through fed batch fermentation with constant feed supply

MATERIALS AND METHODS

Preparation of inoculum

Two proteolytic bacterial cultures, *Pseudomonas putida* and *Staphylococcus aureus*, were procured from Sri Venkateswara University, Tirupati, A.P., India. Inoculum was prepared with addition of 10 ml distilled water mixed with 0.05% Triton X-100 solution to 24 hr old agar slant and was shaken well to obtain homogeneous suspension of above mentioned bacteria separately.

Batch Production

Fermentation was carried out in an Erlenmeyer flask (250ml) with synthetic medium of constituents (g/l) of peptone - 5, sodium chloride - 5, beef extract - 1.5, yeast extract-1.5, KH_2PO_4 – 1.18, and K_2HPO_4 - 0.2. Prepared medium was autoclaved at 121ºC, 15 psi for 15 min. Then the sterilized medium was inoculated with 3% (v/v) *Pseudomonas putida*. Same procedure was repeated for *Staphylococcus aureus* and also for mixed consortium (MC) of equal sizes of *Pseudomonas putida* and *Staphylococcus aureus* respectively. Flasks were transferred to orbital shaker and then agitated at 250 rpm for 48 hr at temperature of 35 ± 2^0C. After required incubation, the flask contents were filtered through Whatman No. 1 filter paper and the filtrate was used for the assay of protease [14, 15] and protein [16]. Dry weight of biomass was determined [17]. Protease concentration was calculated in terms of tyrosine using a standard tyrosine curve and its activity was expressed as µg of tyrosine produced per min per ml of enzyme.

Optimization of process parameters through conventional approaches

In order to investigate the effective inoculum of mixed consortium, alkaline protease production was performed with the size range of 1 – 8 % (v/v). The optimum size of potential bacterial suspension (MC3) was used in further experimentation. The effect of incubation period on enzyme activity was tested by performing the batch fermentation at different time intervals of 12 to 144 hr. Similarly, impact of pH on enzyme synthesis was determined with an initial pH range of 6 to 10 and incubated for 72hr at 35 ± 2^0C. In the same way, temperature was optimized with range 20ºC to 45ºC for incubation of 72 hr with initial pH of production medium at eight.

Role of carbon sources on alkaline protease production

Peels of beetroot, potato, pineapple, and banana were collected from local vegetable market. The peels were cleaned with tap water and dried in hot air oven maintained at 50ºC and then made them into powder using a grinder-mixer and stored in air-tight jars at room temperature till use. The green gram husk was collected from local flour mill. Dried tamarind seeds were collected from local market and ground to powder. In the present study, a total of thirteen carbon sources 2.0% (w/v) such as glucose, fructose, maltose, sucrose, starch, pectin, green gram husk, banana peel, barley powder, beetroot peel, potato peel, pineapple peel, tamarind seed meal were used. Production medium was designed as 2 % (w/v) carbon source, 1% (w/v) yeast extract and 0.5% (w/v) sodium chloride in order to replace the synthetic medium and also to economize the enzyme production. Next to it, optimization of best carbon source, banana peel, was investigated with its concentration range of 1 to 6% (w/v) along with 1% (w/v) yeast extract and 0.5% sodium chloride. Further study was carried out with the optimum concentration of the fruit waste.

Effect of nitrogen sources used for alkaline protease production

Dried cotton seeds were obtained from local market and were ground in mortar with pestle to made it powder. Corn flour and soybean meal were purchased from local super market. Poultry feathers were collected from local farms then washed extensively and boiled. Then it is dried in hot air oven for 4 hr at 50°C. The dried feathers were then

pulverized and the powder was used as feather meal [18]. A total of eight nitrogen sources of 1.0% (w/v) such as yeast extract, urea, peptone, ammonium nitrate, feather meal, soybean meal, cotton seed meal, and corn flour were selected for enzyme synthesis. Further, the best nitrogen source was optimized with various concentration range of 1% to 6% (w/v) with the addition of 2% (w/v) banana peel and 0.5% (w/v) sodium chloride.

Statistical analysis

All the experiments were done in triplicates and the results were presented as mean of these three trials and Standard Deviation (mean ± SD). ANOVA was performed using Microsoft Excel 2007. P values < 0.05 were considered significant with a confidence limit of 95% [13].

Fed batch fermentation

Fermentation medium was prepared with optimized banana peel 2% (w/v), soybean meal 4% (w/v), and sodium chloride 0.5% (w/v). Then autoclaved medium was inoculated with 5% (v/v) of MC3 and incubated at 40ºC with agitation at 150 rpm. Then 10 ml of sterilized medium was supplied intermittently to the bioreactor at a regular interval of 24 h during operation. The fermentation was extended for thirteen days. Sample was taken at regular interval of 24 hr and analyzed for concentrations of fructose, biomass, and the alkaline protease activity. Analysis was performed in triplicates and results were represented as mean ± SD.

RESULTS AND DISCUSSION

Impact of inoculum size on protease production

Size of inoculum is an important biological factor that creates a balance between the biomass and available materials that enhance optimum enzyme production [19]. Shake flask experiments were performed with suspension of *Staphylococcus aureus, Pseudomonas putida* and co-culture separately. Response of the fermentation was summarized in the Table 1.

Table 1: Comparative production of protease from collected bacterial cultures

Culture	Concentration of inoculum (% v/v)	Protease activity(U/ml)
Staphylococcus aureus	3.0	475±0.794
Pseudomonas putida	3.0	541.6±1.999
Mixed culture (MC: equal volumes of *S.aureus and P.putida*)	3.0	675±1.104

**Results indicated in the above table were mean ±SD p<0.05*

From the results, it was observed that the minimum enzyme activity was obtained with *Staphylococcus aureus* alone while the maximum was noticed from MC that was 1.21 times more than *Pseudomonas putida*. Earlier reports revealed that highest protease activity from bacterial species such as *Bacillus subtilis*, *Pseudomonas* sp. *a*nd *Staphylococcus* sp. was around 390±15 U/ml [20].

Below figure1 has exhibited the enzyme activities from MC with the inoculum range of 1- 8 % (v/v). The inoculum magnitude of 5 % (v/v) was superior (901.67±1.156 U/ml) to others.

The effective inoculum size was in good accordance with the industrial inoculum range of 1 to 10% [21]. Moreover the role of different volume ratios of *Pseudomonas putida* and *Staphylococcus aureus* in five percentage of mixed consortium was studied and results were reported in Table 2. These findings were in good agreement with earlier studies [22, 23] for *Bacillus* sp.

From the outcomes of Table 2, MC3 was capable of yielding highest protease activity of 1066.19±28.55 U/ml which was 1.81 more than control (MC1). It was also noticed that the higher volume of *Pseudomonas putida* and lower volume of *Staphylococcus aureus* in MC was the better combination for achieving the significant enzyme activity.

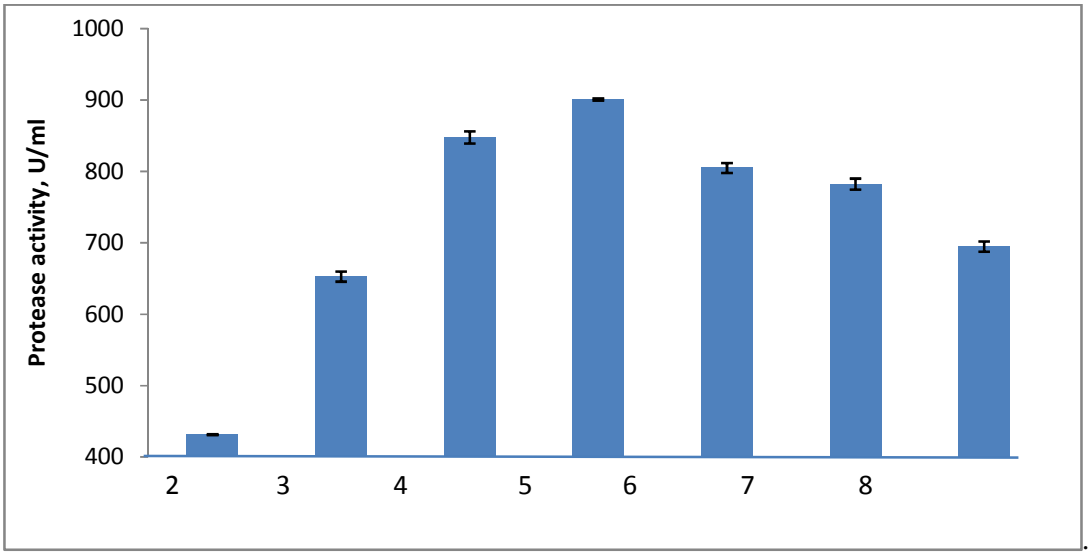

Figure 1: Effect of different concentrations of mixed consortium on protease production

Table 2: Bacterial cultures in mixed consortium for protease production

Mixed consortium	Volume of *P.putida* (ml)	Volume of *S.aureus* (ml)	Protease activity (U/ml)
MC1 (Control)	2.5	2.5	901.67±0.221
MC2	3.0	2.0	1000.16±0.82
MC3	3.5	1.5	1066.19±0.636

**Results indicated in the above table were the mean ±SD, p<0.05.*

Role of temperature, pH and incubation period on protease production

Production and optimization of protease from MC3 was examined through classical approach in batch SmF. Influence of various factors viz. incubation time, initial pH of cultivation medium, and the incubation temperature was investigated.

Incubation period has played an important role in the enzyme production (Table 3). The outcomes of Table 3 have revealed that there was steady increase in enzyme production from 12 hr to 72 hr of incubation.

Table 3: Role of incubation period on enzyme activity

Incubation time (hr)	Protease activity (U/ml)
12	455.17±1.717
24	666.63±0.364
36	814.51±0.28
48	1066.63±0.899
60	1123.14±0.709
72	1184.16±0.561
84	1105.58±0.14
96	833.06±1.414
120	807.09±0.998

**Results indicated in the above table were mean ±SD p<0.05*

The maximum accumulation of protease from MC3 was noticed with incubation time of three days. Slight decline in the protease activity was observed with further incubation which could be due to depletion of essential nutrients. This result has indicated that more amount of protease was produced in later exponential and early stationary phases of growth culture. The maximum activity of protease (1184.16±26.04) was attained at 72 hr of fermentation. Several authors examined that the maximum protease activity was obtained at incubation period of 72 hr with *Bacillus sp.* [22] and *Bacillus thuringiensis* [24]. On the contrary, 48 hr of incubation was reported with *Bacillus laterosporus* for the maximum secretion of protease [25].

The pH of the culture strongly affects many enzymatic processes and transport of compounds across the cell membrane [3] especially concentration of hydrogen significantly affects the protease activity [26]. The effect of medium initial pH in the range of 5.0 to 9.0 was studied in batch SmF for enzyme production at 35±2°C with cultivation time of 72 hr (Figure 2). Peak activity of 1230.62±14.55 U/ml was obtained at pH of 8.0. It was gradually increased from pH 5.0 to 8.0 and was declined at pH more than 8.0. Enzyme synthesis was increased with increase of medium pH towards alkaline range from neutrality, maximum being at pH 8.0 and further increase in pH resulted in decrease of protease production. These findings were coinciding with previous studies [21]. It was noted that pH of the fermentation broth was reduced to alkaline value at the end of incubation. Therefore MC3 is being capable of producing alkaline protease. Similarly findings have reported that effective pH for alkaline protease production from *Bacillus cereus* is in between nine and ten [27 – 29] . Alkaline proteases were largely produced by a gram-negative bacterium, *Pseudomonas* sp [3]. Therefore, in the subsequent experiments, the initial pH of cultivation medium was adjusted to 8.0.

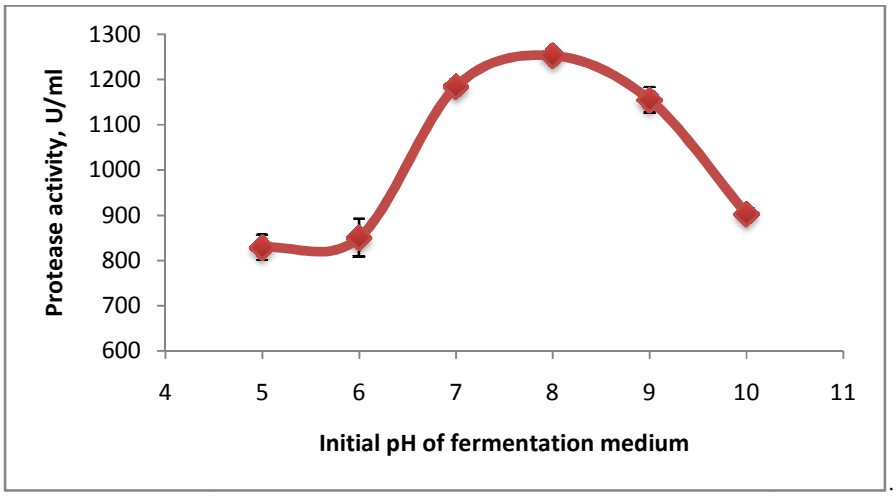

Figure 2: Relation between protease activity and pH of medium (Results are mean ±SD)

The important environmental factor is the incubation temperature as it affects the growth of bacterial cultures and finally alkaline protease productivity. The effect of temperature on alkaline protease production by MC3 was summarized in Table 4. A variety of temperatures was tested in the present study, the effective temperature for the growth and productivity was found to be 40^{0}C for enzyme production. Therefore, bacterial cultures of present experimental study were categorized as mesophilic cultures. The maximum activity was observed at 40^{0}C with *Bacillus laterosporus* [25].

Table 4: Effect of temperature on protease production

Temperature (˚C)	Protease activity (U/ml)
20±2^{0}C	562.9±7.66
25±2^{0}C	744.25±6.661
30±2^{0}C	934.1±0.169
35±2^{0}C (Control)	1230.62±20.57
40^{0}C	**1306.3±14.269**
45^{0}C	1140.6±3.719

**Values in table are represented as mean ± SD (P<0.05)*

The effective temperature range for alkaline protease production from *Bacillus* isolates was in between 30^{0}C - 50^{0}C [12] while a temperature of 75^{0}C was the optimum for bacteria isolated from an alkaline hot spring[14]. The maximum protease activity at 45^{0}C with *Bacillus* sp [22]. An optimal pH and temperature of the protease activity from *Bacillus licheniformis* was reported as 9.00 and 50^{0}C [29]. Authors also reported that protease activity was found to reduced at 60^{0}C and the enzyme was unstable at this highest temperature [29]. However, maximum enzyme activity from *Bacillus thuringiensis* was reported at lowest incubation temperature 30^{0}C [24].

Role of carbon sources on alkaline protease production

Supplementation of fermentation media with a suitable carbon enhances the enzyme productivity irrespective of the type of fermentation [7,17]. In this connection, the effect of different carbon sources (2% w/v) was tested on the alkaline protease production from MC3 at an initial pH of 8.0, a temperature of 40^0C and with an agitation of 150 rpm for 72 hr of incubation (Figure 3). Protease synthesis was repressed by glucose, maltose, fructose, green gram husk, barley powder, beetroot peel, potato peel, sucrose, pectin, starch and pineapple peel while stimulated by tamarind seed meal, and banana peel**.** The highest activity of 1433.39 U/ml was given by the MC3 from banana peel followed by tamarind seed meal where as the minimum of 100 U/ml was resulted with glucose. Among the tested monosaccharides, fructose was better than glucose for enzyme yield. The main carbohydrate of banana peel is fructose, which was resulted in the highest protease activity under aforesaid conditions. Therefore, fermentation medium was designed with banana peel as the main carbon source thereby the process was economized for enzyme production.

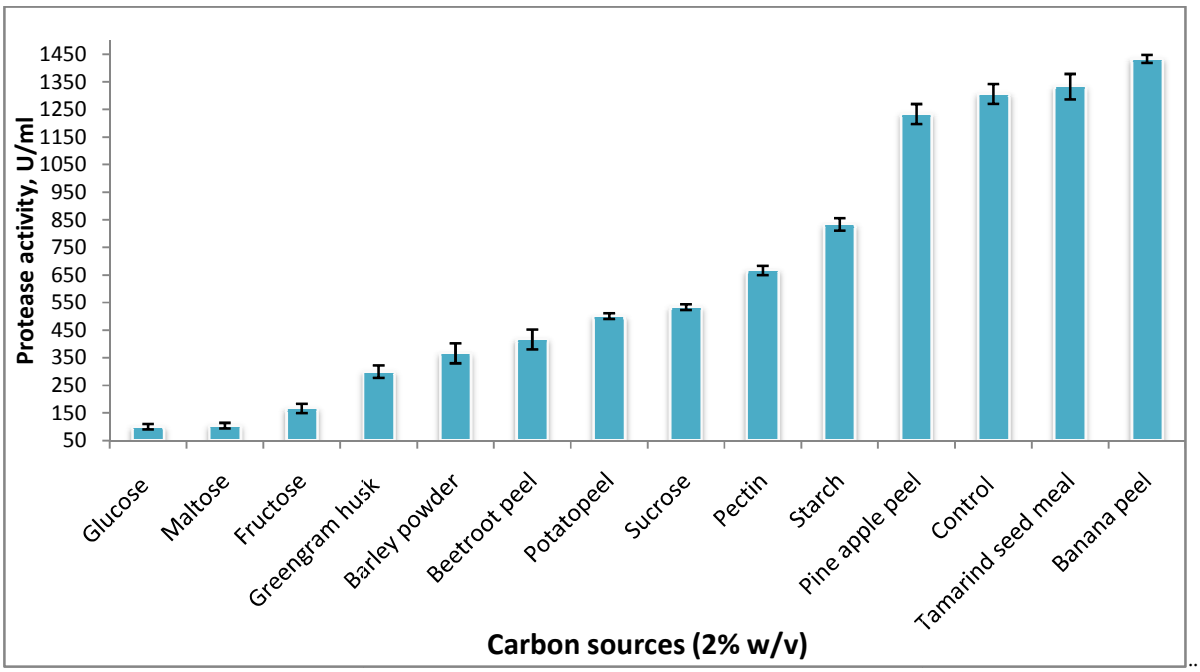

Figure 3: Effect of various carbon sources on protease activity

Pure form of disaccharide, sucrose, was better than glucose, fructose, and maltose. In contrast, highest protease activity from *Bacillus* sp. was achieved with sucrose [27]. Another report made that maltose was the effective carbon source for alkaline protease synthesis from *Pseudomonas aeruginosa* isolated from dairy effluent sludge [13]. Maximal activity (1433. 67±39.598 U/ml) was achieved with 2% (w/v) banana peel while greater concentrations (> 2% (w/v)) were shown inhibitory to the productivity of enzyme and also to the cell growth (Figure 4).

Effect of different nitrogen sources on alkaline protease production

Nitrogen source is metabolized to produce primarily amino acid, nucleic acid, protein and cell wall components. These nitrogen sources have regulatory effect on the enzyme synthesis. Production of protease is highly dependent on both carbon and nitrogen sources available in the medium [13]. The impact of different inorganic, organic and agro based nitrogen sources on biomass and alkaline protease production from MC3 by utilizing banana peel (2% w/v) as the main carbon source were shown in Table 5.

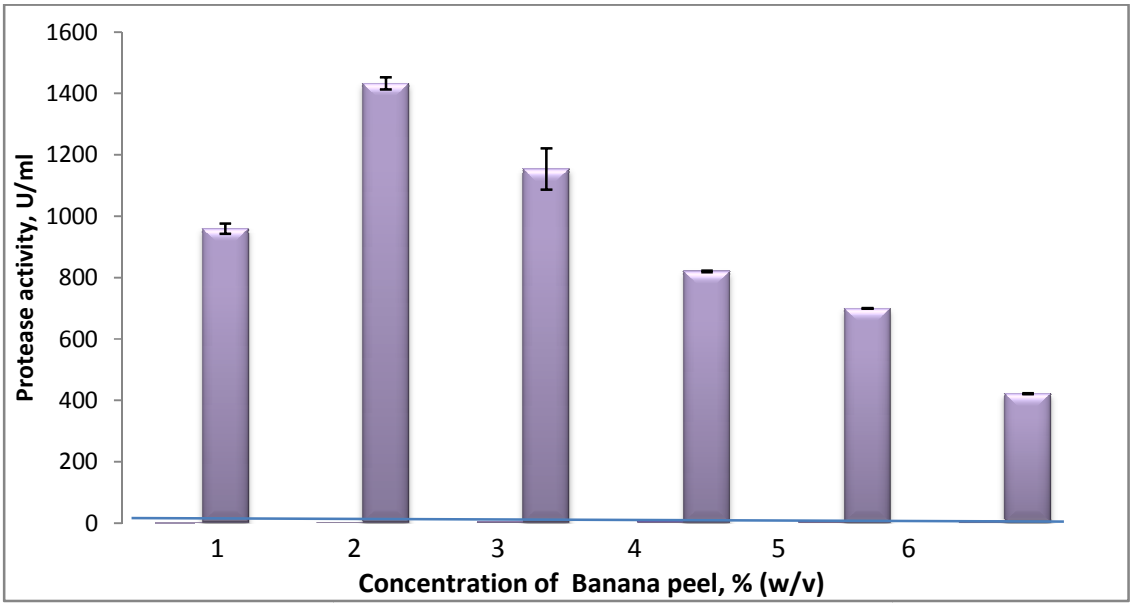

Figure 4: Relation between enzyme activity and amount of banana peel

Table 5 : Role of various nitrogen sources on alkaline protease activity

Nitrogen source (1%w/v)	Protease activity (U/ml)
Yeast Extract (Control)	1433±39.598
Feather meal	233.03±15.019
Corn flour	333.06±15.302
Cotton seed meal	433.12±16.433
Peptone	700.15±18.964
Ammonium nitrate	633.37±17.098
Urea	900.20±19.799
Soybean meal	1419.81±2.999

Fermentation medium supplemented with yeast extract (control) resulted in the highest activity of 1433.91 ± 14.23 U/ml. In order to design low cost medium, second best nitrogen source, soybean meal, was considered as the suitable nitrogen. Maximum activity was obtained with yeast extract followed by soybean meal. Alkaline protease activity range of 230 to 900 U/ml was observed with other nitrogen sources tested in the present experimental study. Low cost agro industry based nitrogen source, soybean meal was shown to considerable impact on protease synthesis (Enzyme activity of 1419.81±2.999 U/ml) while the remaining were found to repress the enzyme activity. Based on the economic point of view, soybean meal was considered as the suitable nitrogen source for enhanced alkaline protease activity. An experimental study reported that yeast extract was the best nitrogen source for alkaline protease production from *Pseudomonas aeruginosa* [13]. A decrease trend in enzyme activity was found with soybean meal concentrations more than four percent (Figure 5).

Modeling of alkaline protease activity from mixed consortium

Simple polynomial models were proposed for extracellular alkaline protease in submerged fermentation with the help of software, MATLAB. With the below formulated models, alkaline protease activity can be predicted at any pH, incubation time. Similar models for acid protease production from *Aspergillus* sp. under solid state fermentation were proposed [17].

$$\mathbf{Y\ (I) = -0.196I^5+6.199I^4-65.799\ I^3+\ \ 254.135I^2\ -112.097\ I+\ 42.464} \qquad \mathbf{(4.1)}$$

Where Y (t): Protease activity as a function of Inoculum size, I, U/ml

$$\mathbf{Y\ (t) = 0.0001t^4-\ 0.0201\ t^3+\ 1.4457\ t^2\ -\ 21.6749\ t+\ 557.522} \qquad \mathbf{(4.2)}$$

Where Y (t): Protease activity as a function of incubation time, t, U/ml

$$Y(pH) = -2\,pH^5 + 82pH^4 - 1448pH3 + 12314\,(pH)^2 - 50452\,(pH) + 80418 \quad (4.3)$$

Where Y (pH): Protease activity as a function of initial pH of medium, U/ml

$$Y(T) = 0.22T^3 + 22T^2 - 627.91\,T + 6254.4 \quad (4.4)$$

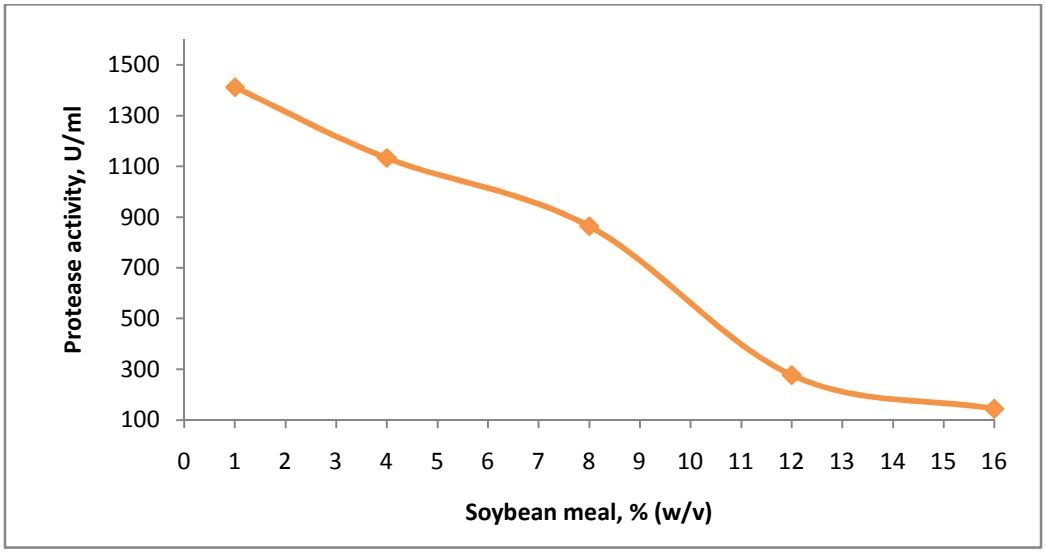

Figure 5: Role of various concentrations of soybean meal

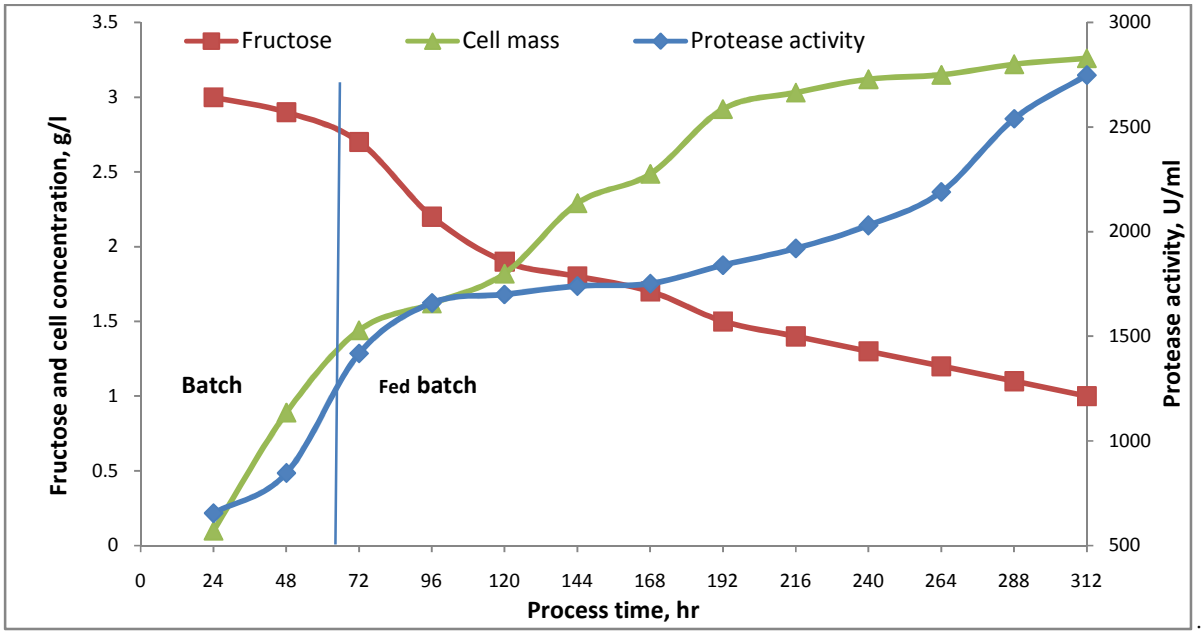

Figure 6: Kinetics of fructose, protease activity, and biomass

Fed batch fermentation

In general fed batch fermentation is used in order to reduce the inhibitory of either carbon or nitrogen sources thereby enhancing the productivity of desired metabolite [9,11,30]. Therefore cultivation was shifted to fed batch

from batch with constant feeding strategy of carbon and nitrogen sources at the end of 70 hr of incubation. During the process it was difficult to determine the concentration of soybean meal. Consequently, fructose of banana peel, protease activity, and bacterial concentration were analyzed at regular interval of 24 hr and out comes were shown in Figure 6. The vertical solid line denoted the start of intermittent supply of sterilized medium. At the end of batch cultivation, concentration of biomass was found to be 3.26 g/l. A low concentration of fructose was maintained during the entire fed-batch operation. Final enzyme activity achieved as 2748 U/ml which was 1.94 fold - increase than batch. Increased trend of activity was noticed during fed batch cultivation. Biomass of mixed consortium grew continuously up to 192 hr of operation and thereafter it was almost constant around 3.13 g /l. Moreover, soybean meal concentration of 4% w/v was found to be inhibitory from batch which was resulted in enhanced biomass growth and enzyme synthesis. Production of protease and biomass were progressed because of supply of carbon and nitrogen sources during fed-batch cultivation.

Specific growth rate, μ, was computed by using $\mu = \frac{1}{X}\frac{dX}{dt}$. During fed batch fermentation, it was found to be reduced continuously (Figure 7).

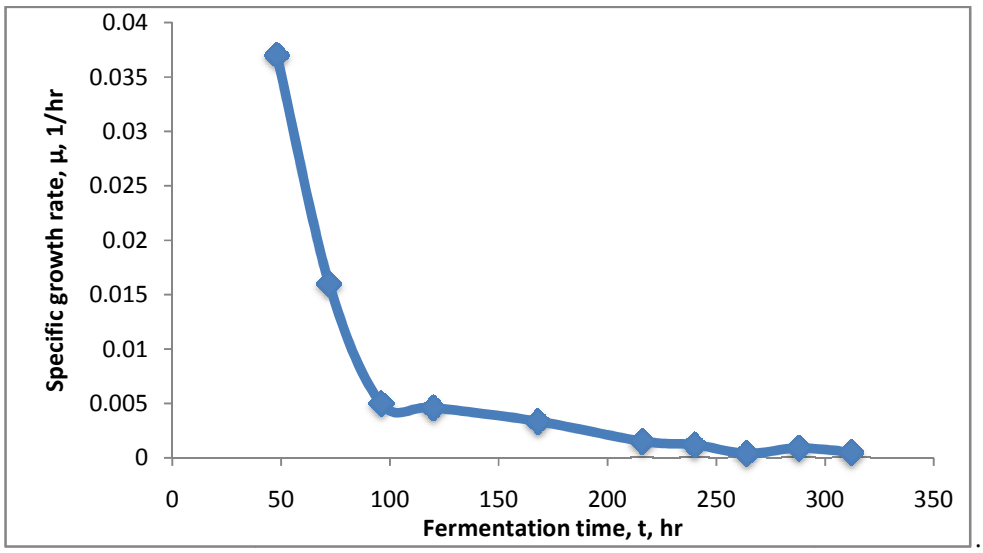

Figure 7: Relation between fermentation time and specific growth rate

CONCLUSION

Coculture of *pseudomonas putida and Staphylococcus aureus* could produce alkaline protease using banana peel as the main carbon source through submerged fermentation. Long –term incubations were possible with fed batch mode of operation with constant supply of banana peel and soybean meal. Higher concentration of soybean meal (≥ 4.0% w/v) was inhibitory to protease production which was lessend by fed batch cultivation. Enhanced enzyme activity was achieved through fed batch than batch process. In future, various feeding strategies of fed batch are to be investigated.

Acknowledgements
Authors express their gratitude to Sree Vidyanikethan Engineering College to provide the laboratory facilities to carry out present experimental work**.**

REFERENCES

[1] http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/4.
[2] M,B, Rao, A,M, Tanksale, M,S, Ghatge, V,V, Deshpande , *Microbiology and Molecular Biology Reviews*., **1998**, 62(3), 597-635.
[3] S,T, Vasantha, A,T,Subramanian, *International Current Pharmaceutical Journal*., **2012**, 2(1), 1-6.

[4] 4.http://www.marketresearchreports.biz/analysis-details/global-industrial-enzymes-market-report-**2013**.
[5] N, Akcan,F, Uyar, *Eurasian Journal of Biosciences*., **2011**, 5, 64-72.
[6] P, Singhal, K,V,Nigam, S, Vidyarthi, *International Journal of Advanced Biotechnology and Research*, 3(3), 653-669, (2012).
[7] S, Radha, V,J, Nithya, R, Himakiran Babu, A, Sridevi, N,B,L, Prasad,G, Narasimha, *Archives of Applied Science Research*., **2011**, 3(2), 155-163.
[8] F, Tabandeh, H, Moghaddam, H, Mousavian,B,Yakhchali, *The 11th Iranian Chemical Engineering Congress (ICHEC11)*, **2006**, 28-30.
[9] L,M, Shuler, F, Kargi , Second edition, PHI, **2003**,276-281.
[10] U, Beshay, *Chem. Biochem. Eng.*, **2008**, *Q*.22 (1), 119–124.
[11] Chongyang Liu , *Applied Mathematical Modelling*, **2013**,37, 695–706.
[12] A,K,Lawal , S,O,Olatope , Y,L,Majolagbe, F,A,Alebiosu, J,B,Bashar , O,F, Kayode, E,N,Dike , S,O,Akinola, G,N, Elemo, *Prime Journal of Microbiology Research.*, **2011**, 1(2), 27-37.
[13] Amrita Raj, Nancy Khess, Namrata Pujari, Sourav Bhattacharya, Arijit Das, Subbaramiah Sundara Rajan, *Asian Pacific Journal of Tropical Biomedicine.*, **2012**,S1485, 1845-1851.
[14] P, Wilson, Z, Remigio, *African Journal of Microbiology Research.*, **2012**,5542-5551.
[15] S,Radha, R,Himakiran Babu, A, Sridevi, N, B, L, Prasad,G, Narasimha, *European Journal of Experimental Biology*., **2012**, 2 (5), 1517-1528.
[16] O,H, Lowry, N,J, Rosebrough, A,L, Farr,R,J,Randall, *J. Biol. Chem.*, **1951**,193(1), 65- 75.
[17] S,Radha, R,Himakiran Babu, A, Sridevi, N, B, L, Prasad,G, Narasimha, *J. Microbiol. Biotech. Research*., **2012**, 2 (1), 6-16.
[18] A, Sarita, W, Neeraj , *International Journal of Poultry Science*., **2010**, 9 (5), 482-489.
[19] C, Sandhya, A, Sumantha , G, Szakacs G, A, Pandey, *Process Biochemistry.*, **2005**,40, 2689–2694.
[20] K, M, Siva Muthuprakash, A, Jayanthi, *International Journal of Science and Nature*., **2011**, 2(1), 110-113.
[21] P,F, Stanbury, A, Whitaker, S,J,Hall, Second Edition , Butterworth-Heinemann Publications., **2003**, ISBN 07506 45016.
[22] G,U,O**,** Okafor, M,E,E, Anosike, *Research Journal of Microbiology.*, **2012**, 7, 327-336.
[23] R**,** Abusham, Raja Noor Zaliha, Rahman, Abu Bakar Salleh, Mahiran Basri, *Microbial Cell Factories.* , **2009**, 8-20.
[24] M,S, Foda, M,S, Ali, A,M,Youssef, T, Kahil, H, M. Shata, A, M. Roshdy, *Journal of Applied Sciences Research.*, **2013**, 9(3), 1975-1984..
[25] B, Usharani, M, Muthuraj , *African Journal of Microbiology Research.*, **2010**, 1057-1063.
[26] M,M, I, Helal, A, Hassan, A,M, Nayera, Abdelwahed, Madeha, O,I,Ghobashy, *Australian Journal of Basic and Applied Sciences*., **2012**, 6(3), 193-203.
[27] T. Kuberan, S, Sangaralingam, V,Thirumalai Arasu, *Journal of Biosciences Research*., **2010**, 1(3), 163-174.
[28] G, M, Abou-Elela, A,H, Hassan, S, W, Hassan, H, Abd-Elnaby, N, M. K. El-Toukhy, *African Journal of Biotechnology.*, **2011**, 10(22), 4631-4642.
[29] M, Anvari, G, Khayati, *Trends in Applied Sciences Research* .,**2011**, 6(10), 1206-1213.
[30] U, Beshay, A, Moreira A, *Biotechnology Letters*.,**2005**, 27: 1457-1460, (2005).