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Optimal Conditions for Production of Extracellular Alkaline Protease from Newly Isolated *Bacillus* Sp from Leather Industry Effluents

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ABSTRACT

The growth and protease production by *Bacillus* sp.was examined for leather processing industries. The maximum protease activity was 88 mg/ml using 4% (w/v) of Mongdal powder as substrate. Mongdal powder is an inexpensive and readily available, thus it can be used as the cost effective crude material for the production of an extracellular protease. Inorganic nitrogen sources proved to be less favorable, for protease production as strong catabolic repression was observed with ammonium ions. A maximum of 36 mg/mL of protease was produced in 48 h in a 22 L bioreactor in addition, the enzyme was found to be very stable toward of the metal ions used (Ca^{2+}, Mg^{2+}, KH_2PO_4, and NaCl) maximum enzyme activity found in KH_2PO_4 32 mg/ml.

Keywards: Alkaline protease,Mongdal powder, *Bacillus* sp., protease, soybean.

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INTRODUCTION

Biocatalysts that perform a multitude of chemical reactions and are commercially exploited in the detergent, food, pharmaceutical, diagnostics, and fine chemical industries. Of the 3000 different enzymes described to date the majority have been isolated from mesophilic organisms. These enzymes mainly function in a narrow range of pH, temperature, and ionic strength. Moreover, the technological application of enzymes under demanding industrial conditions makes the currently known arsenal of enzyme not recommendable. Thus, the search for new microbial sources is a continual exercise, but one must respect biodiversity. The microorganisms from diverse and exotic environments, (Extremophiles) are considered as important source of enzymes, and their specific properties are expected to result in novel process applications (Yoshida *et, al,*. 1991). Alkaline proteases (*Bacillus subtilis*, E.C. 3.4.21.14) are a physiologically and commercially important group of enzymes used primarily as detergent additives. They play a specific catalytic role in the hydrolysis of proteins. In 1994, the total market for industrial enzymes accounted for approximately $400 million, of which enzymes worth $112 million were used for detergent purposes. In Japan, 1994 alkaline protease sales were estimated at 15000 million yen (equivalent to $116 million) (Shimogaki H*et al.*, 1991)

Isolation of Alkalophiles (Alkaline Bacteria)

The sample was collected from the sheep upper collagen layer of skin which is obtained from the

1. S.A.Tannery of Erode, Tamil Nadu.
2. Naser Tanning company of Chennai, Tamil Nadu.
3. A.M.Sadick tanners of Bhavani, Tamil Nadu and
4. KKSK Leather Processor of Trichy, Tamil Nadu.

About 1.0g of skin sample was transferred to 99.0 ml sterilized normal saline in 250 ml conical flask and agitated (100 rpm) at 37° C for 1 hour on shaker.

The skin suspension was then diluted in serial up to 10^{-7} dilution. One ml of each dilution was poured into petri plates containing nutrient agar medium of pH 9. The inoculated plates were incubated at 37°C for 24 hours.

Screening of Bacterial Alkalophilies

Individual bacterial colonies were screened for proteolytic enzymes production on skim milk agar medium, containing skim milk 1.0%, peptone 0.5%, sodium chloride 5% and agar 2.5%. The pH of the medium was adjusted to 9 with 1 N HCL/ 1 N NaOHbefore sterilization at 120°C for 15

minute. The inoculated plates were then incubated at 37°C for 48 hours and observed for zones of clearance which indicate proteolytic activities.

Identification of the Proteolytic Isolates

The bacterial isolates with prominent zones of clearance were processed for the determination of morphology, gram characteristics, motility, citrate utilization, oxidase, urease, gelatin liquidation, catalase, VP, and indole tests, acid production from D-Glucose, D-Arabinose, D-Lactose, D-Mannitol, D-Galactose and D-Maltose. The isolates were also grown at different temperature, pH and NaCl concentration. These isolates were then identified in accordance with the methods recommended in Bergey's Manual of Determinative Bacteriology. (John. Holt,Lippincott and Wilkins 1994).

Enzyme Production Medium

Production medium contained (g/l) glucose 0.5gm,peptone 0.15gm,$Feso_4$ 0.1gm, KH_2PO_4 0.5gm,$MgSo_4$ 5gm 10ml of medium was taken in 100ml conical flask. The flasks were sterilized in autoclave at 121ºC for 15 min and faster cooling the flask was inoculated with overnight grown bacterial culture. The inoculated medium was incubated at 37ºC in shaker incubator for 24 hours. At the end of the fermentation period, the culture mediumwas centrifuged at 5000 rpm for 15 min to obtain the crude extract which served as enzyme source.

Protease assay

Protease was determined by the method of Folin Lowry Method. Protease activity is assayed by using 1ml of 1% casein in 0.05M Tris HCL Buffer(pH7.8) as substrate. Casein solution is incubated with 0.5ml of enzyme at 50C for 30 minutes. After 30 minutes, the reaction is terminated by the addition of 2ml of 10%TCA. Mixture is centrifuged and 1 ml of supernatant was added to 5 ml alkaline reagent. This is preceded by the addition of 0.5ml of Folinciocalteau reagent. After 25-30 minutes the colour developed is read at 540 nm against a reagent blank prepared in the manner. One unit of prease activity is defined as the amount of proetease which liberate 1µg of tyrosine experimental condition.

Process optimization of Maximum protease production

1. Effect of supplementary organic nitrogen source on protease production

The effect of various organic nitrogen sources such as Bengal gram powder(A), Black gram powder(B), soya bean meal, Green gram powdered, De-Fatted soya flour, Full fat soya flour, Ground nut meal, wheat gluten, soya flour isolate(90%) protein, Soya flour hydrolystate, Bengal gram powder (Tostate),Soya flour (de fatted) tosted, Mongdal powder, Toor dal, Kidney bean, Cow pea, Pearl millet, Finger miller.

2. Effect of amount of substrate on protease production

Differenetquantites of substrate (%w/v) 2,3,4,5,8,10,15 and 20 were submerged in the fermentation medium to investigate the influence of the amount of substrate on the magnitude of enzyme production. The minimum quantity of substrate yielding maximum enzyme was selected for further experiments.

3. Effect of particle size of substrate on protease production

The substrate was sieved throught various mesh size to obtain fine medium,coarse and large particles of<425µm,425-600 µm,600-1000 µm and 1000-1425 µm size respectively to see whether the particle size has any influence of enzyme production.

4. Effect of supplementary carbon sources on protease production

Rrequirement of additional nutrient supply was studied, adding different supplementarycarbon sources (5%w/w)like Glucose,Sucrose,Maltose,lactose,glycerol,starch,mannose and molasses to the fermentation medium. The concentration of 1 to 8% was examined in the production medium.

5. Effect of supplementary inorganic nitrogen sources on protease production

Various inorganic nitrogen sources (0.5%w/w) like Urea, sodium nitrate and ammonium sulphate were examined for their effect on protease production at maximum level of 3 %w/w in the medium.

6. Effect of Supplementary Divalent metal ion sources on protease production

Influence of various metal ions on protease production was determined by incubating the medum with different metal ions such as $CaCl_2$, $MgSO_4$, KH_2PO_4, NaCl, KCL.

RESULTS AND DISCUSSIONS

Effect of supplementary organic nitrogen sources on protease production

Production of extracellular proteases has been shown to be sensitive to repression by different organic nitrogen sources. The effect of organic nitrogen sources was studied in the growth medium. Among the various nitrogen sources tested, Mongdal powder was found to be the best organic nitrogen source for alkaline protease production (Table 1). Various concentrations of organic nitrogen source were used to different percentage like as 2%, 3%, 4%, 5%, 8%, 10%. Result obtained were showed that 8% Mongdal powder brought the highest protease production compared to other percentage (Figure 1). Same results were obtained (Kumar, Takagi 1999), (Cowan 1996).

Table 1 : Effect of supplementary organic nitrogen sources on protease production.

ORGANIC NITROGEN	S3	N2	N4	K2
A	10	21	22	12
B	8	20	21	16
C	7	2	14	19
D	21	4	21	22
E	8	8	18	12
F	6	21	16	6
G	6	26	12	5
H	8	23	17	2
I	12	11	26	7
J	21	12	31	4
K	12	12	35	22
L	12	12	32	12
M	41	24	93	48
N	12	6	22	11
O	6	6	21	16
P	10	23	20	13
Q	8	2	12	21
R	11	31	15	12

THE NUMBERS INDICATE ALKALINE PROTEASE ACTIVITY = MG/ML

Bengal gram powder(A), Black gram powder(B), soya bean meal(C), Green gram powdered(D), De-Fatted soya flour(E), Full fat soya flour(F), Grounut meal(G), wheat gluten(H), soya flour isolate(90%) protein(I), Soya flour hydrolystate(J), Bengal gram powder (Tostate)(K),Soya flour (de fatted) tosted(L), Mongdal powder(M), Toor dal(N), Kidney bean(O), Cow pea(P),Pearl millet(Q),Finger miller(R).

S3:-Bacterial Colonies Isolated from Leather Sample of S. A. Tannery-Erode

N2:-Bacterial Colonies Isolated from Leather Sample of Nasser Tanning Company-chennai

N3:-Bacterial Colonies Isolated from Leather Sample of Nasser Tanning Company-Chennai

K2:-Bacterial Colonies Isolated from Leather Sample of KKSK Leather Processer (P)Ltd-Trichy.

Effect of Particle Size of Substrate on Protease Production

Maximum protease production was obtained with substrate particles of average size<425µm (Figure 2). Shows te results obtained when fermentation was carried out with different particle size of the substrate of Mongdal powder. Larger particles exhibited a decreasing trend towards enzymes yield.

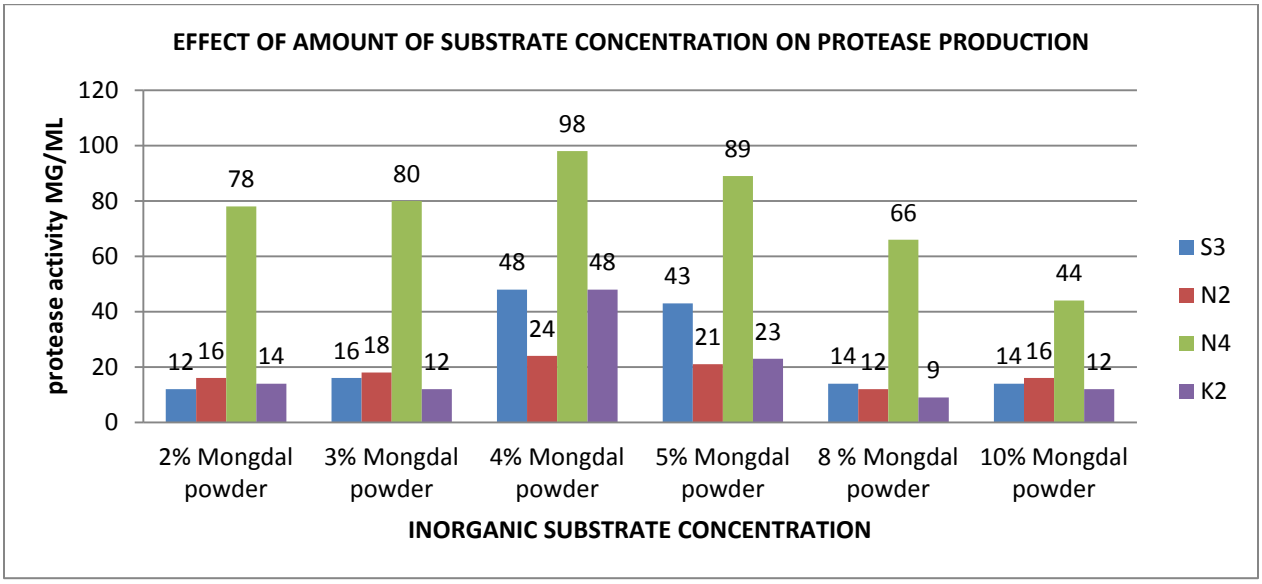

Figure 1: Effect of Amount of Substrate Concentration on Protease Production

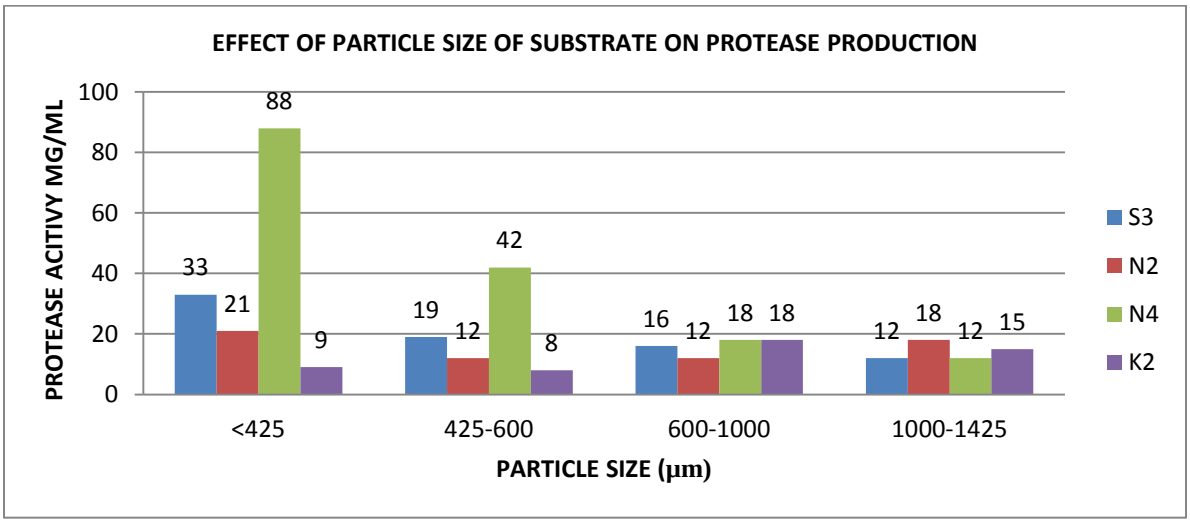

Figure 2: Effect of Particle Size of Substrate on Protease Production

Effect of supplementary carbon sources on protease production

Various sources of carbon such as Glucose, Sucrose, Maltose, Lactose, Glycerol, Starch, Mannose and Molasses. The effect of carbon source on protease production was studied in the growth medium. Among the various carbon source tested. Sucrose was found to be the best carbon source for alkaline protease production(Figure 3).Various concentration of carbon source were used to 2%,4%,6%,8%. 6% Sucrose Carbon source brought the highest protease production compared to other percentage carbon sources (Figure 4).Similar results have been reported by (Gupta *et al.*, 2002) ,(Rao *et.al.*,1998) and (Horikoshi .1999)

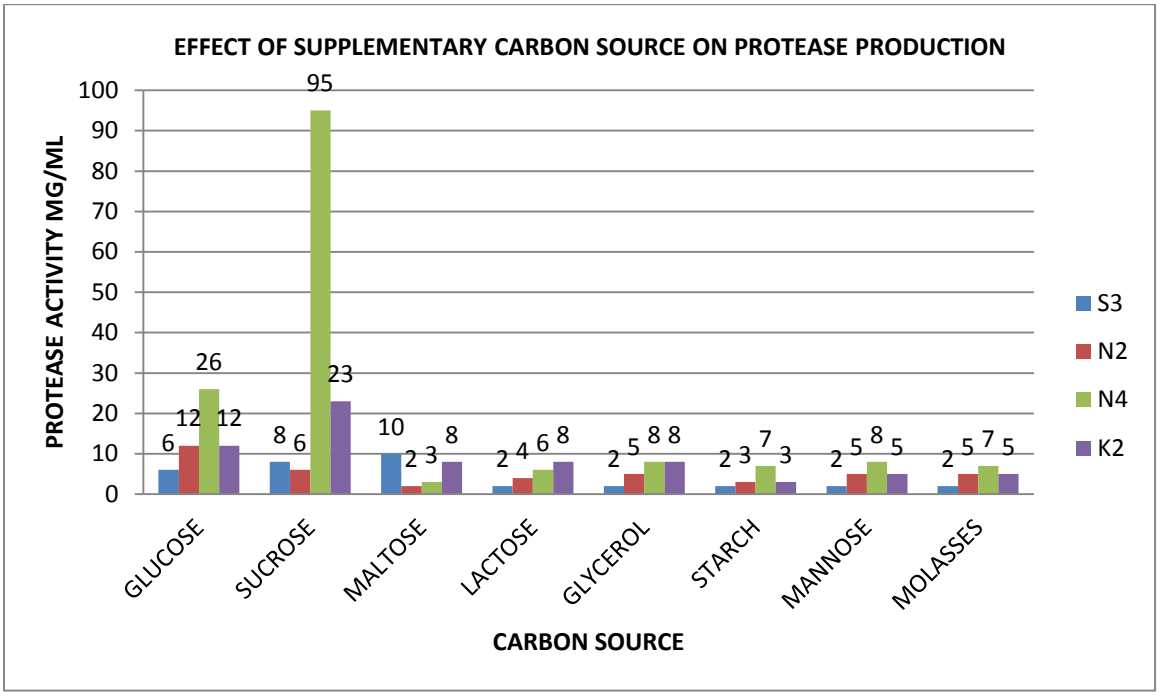

Figure 3: Effect of Supplementary Carbon Source on Protease Production

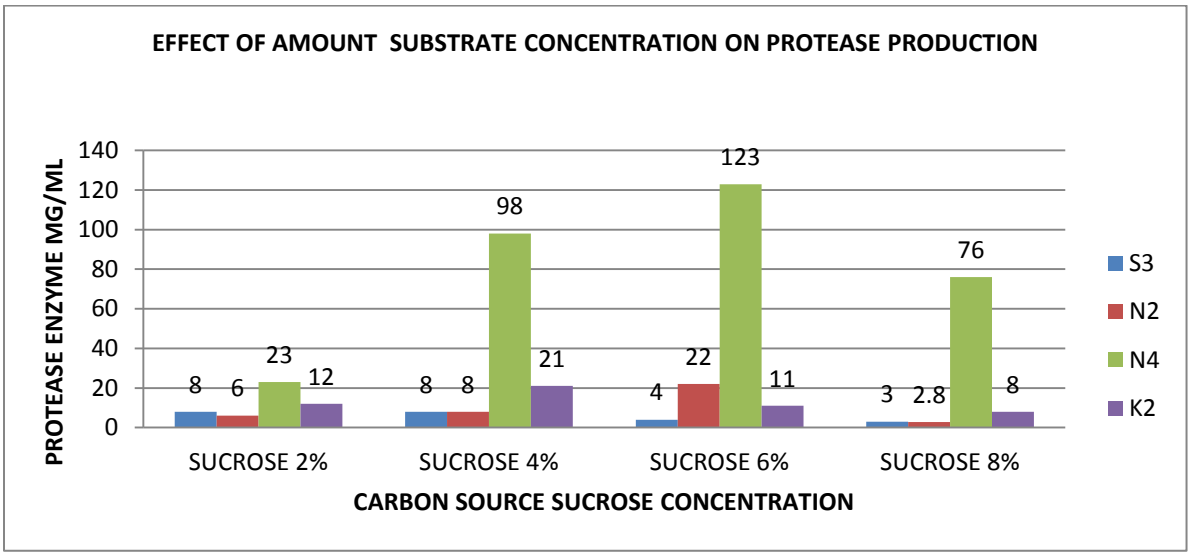

Figure 4: Effect of Amount Substrate Concentration on Protease Production

Effect of Supplementary Inorganic Nitrogen Source on Protease Production

Alkaline protease production were carried out in medium Urea, Sodium Nitrate, Ammonium sulphate and Ammonium chloride various nitrogen sources by replacing only peptone (Figure 5). It is observed that alkaline ptrotease production was appreciably good in all cases but maximum observed that alkaline protease production was obtained using Ammonium sulphate.The maximum production observed in the N4 culture. Various concentration of inorganic nitrogen sources were used to 0.2%,0.4%,0.6%,0.8%,1%,2%. 1% of Ammounium sulphate brought the highest protease

production compared to other percentage inorganic nitrogen sources (Figure 6). (Saeki *et. al.*, 2007), (Moreira *et.al.*, 2002), (Horikoshi, Akiba 1982

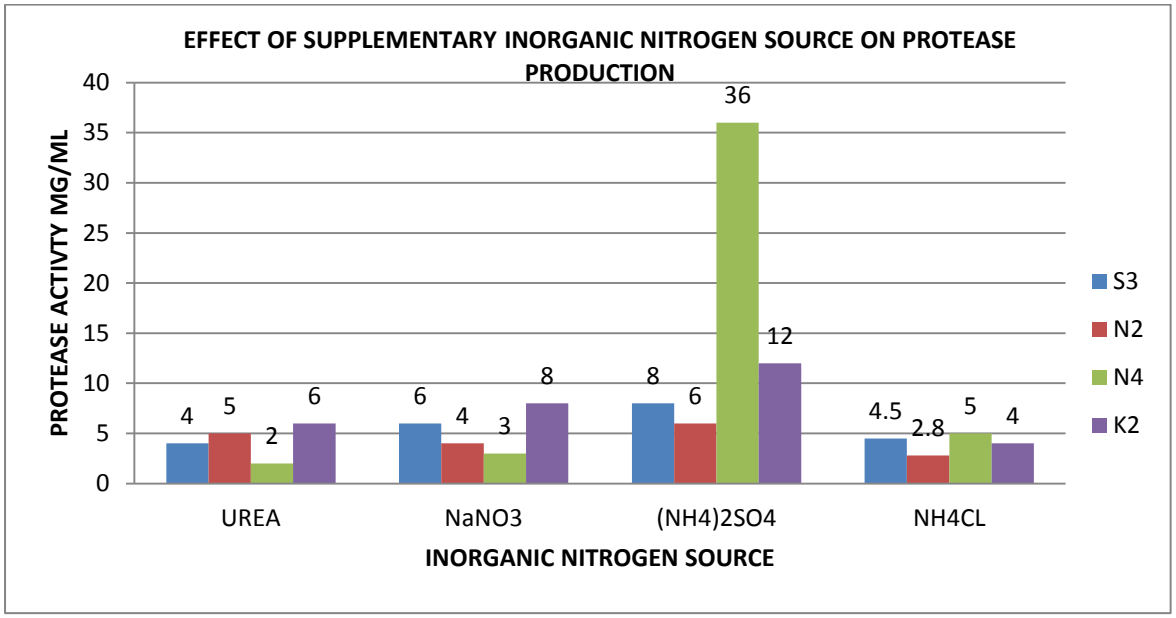

Figure 5: Effect of Supplementarty Inorganic Nitrogen Source on Protease Production

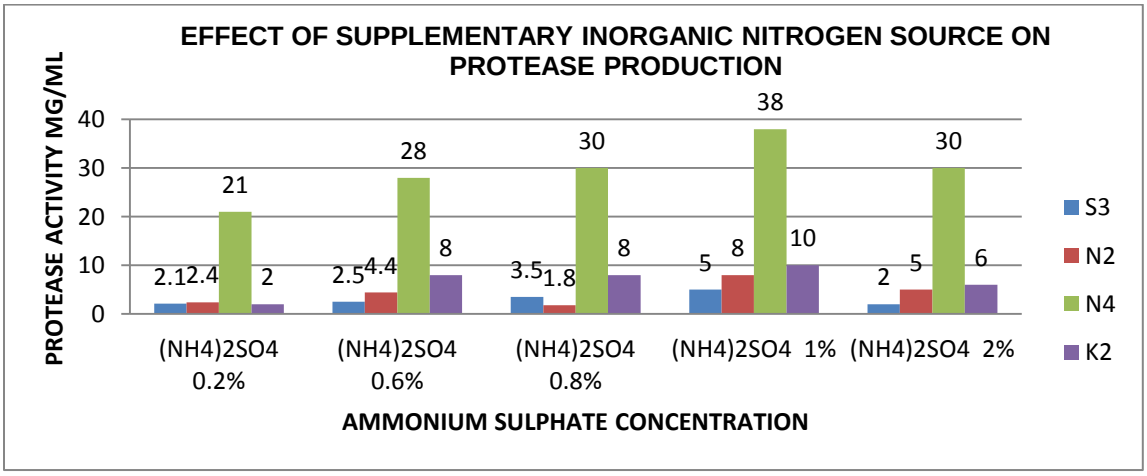

Figure 6: Effect of Supplementary Inorganic Nitrogen Source on Protease Production

Effect of Metal Ions

The metal ions that were used are categorized into activators (Ca^{2+}), as shown in Figure 7, Figure 8). KH_2PO_4 increases the protease activity by preventing it from denaturing at higher temperature. The metal ion concentration decreases in yield may have been due to the fact that denaturation or degradation of the proteolytic enzyme by autolysis in response to elevated metal ions caused alkaline protease activity to decrease (Wan *et. al.*, 2009)(Saeki *et. al*,2007) and (Prakash*et. al.*,2005).

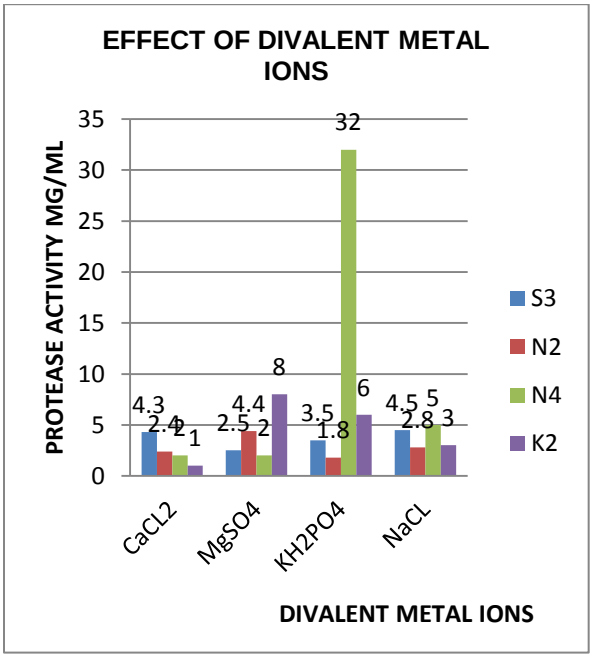

Figure: 7 Effect of Divalent Metal Ions and Concentration

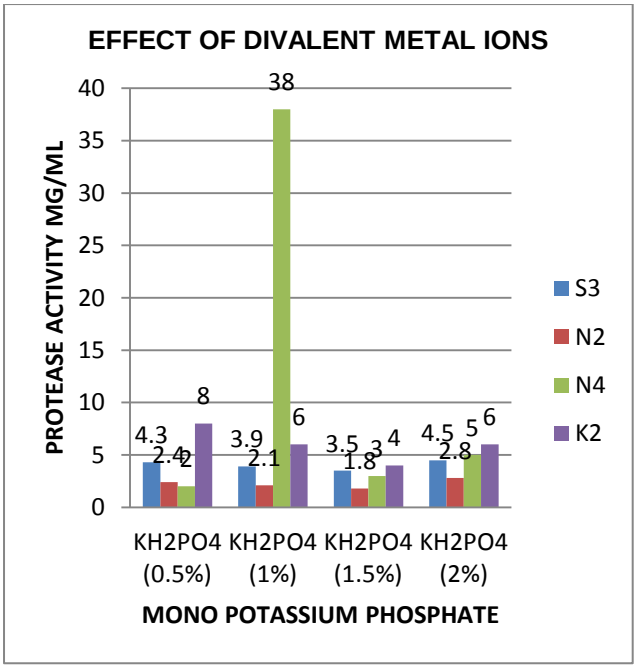

Figure:8 Effect of Divalent ions and protease activity

CONCLUSION

The protease from *Bacillussp*was optimized for its maximum production. The effect of metal ions on its activity was also studied. Purified enzyme was obtained using ammonium sulphate precipitation. It can be effectively used with the commercial detergents.

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