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Production and Optimization of Extracellular Alkaline Protease by *Bacillus subtilis* in Submerged Fermentation

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Abstract

The bacterial strain (Bacillus subtilis) was isolated from soil sample using different composed culture for alkaline protease production in Submerged Fermentationby. The eight different submereged media were prepared to study of enzyme activity. Then the effect of pH, carbon sources nitrogen sources, Nacl concentration and metal ions on protease production were studied. The results indicated to: MD8 produced highest amount of alkaline protease (62.5 U /ml), The extracted crude enzyme was precipitated using varied precentages of ammonium sulphate was studied. The effect of pH,carbon,nitrogen sources sodium chloride concentration and metal ions was studied here and results tom maximum enzyme activity was as pH11 to MD4(17.97U/ml),glucose showed maximum yield in MD2(49.21U/ml) ammonium chloride affected maximum (132.81U/ml) to MD6 and the maximum activity was seen in case of MD5 with 11%NaCl(46.09U/ml) and lastly the metal ions effect was $Mncl_2$(85.94U/ml).

Key word: alkaline protease, *Bacillus subtilis* amd Submerged State Fermentation

الملخص

تم عزل السلالة البكتيرية (*Bacillus subtilis*) من عينة التربة باستخدام بيئات مختلفة التركيب لإنتاج إنزيم البروتيز القلوي في التخمير المغمورللبيئات السائلة. تم إعداد ثمانية بيئات مختلفة لدراسة نشاط الإنزيم. حيث تم دراسة تأثير درجة الحموضة ، وتاثير مصادر النيتروجين و مصادر الكربون ، وتركيز كلوريد Nacl وأيونات المعادن على إنتاج البروتيز. تشير النتائج إلى أن: MD8 أنتجت أعلى كمية من البروتيز القلوي (U / ml 62.5)، وقد تم ترسيب الإنزيم الخام المستخرج باستخدام درجات مختلفة من كبريتات الأمونيوم.

تمت دراسة تأثير الأس الهيدروجيني، الكربون، النيتروجين، تركيز كلوريد الصوديوم، أيونات المعادن .ونتائج نشاط إنزيم توم الأقصى كان عند pH11 في MD4 (17.97U / ml)، أظهر الغلوكوز أقصى إنتاج في MD2 (49.21U / ml)، كلوريد الأمونيوم كان الحد الأقصى في MD6 (32.8U/ml) وكان الحد الأقصى للنشاط في MD5 مع 11 ٪ كلوريد الصوديوم (46.09U/ml) وأخيرا كان تأثير أيونات المعادن وهو كلوريد المنجنيز .(85.94U/ml)

الكلمات المفتاحية :البروتياز القلوي، الباسيلس سبتايلس، بيئة التخمير السائلة

Introduction:

Proteases are one of the most important industrial enzymes produced by wide range of microorganisms such as bacteria, yeasts, molds, and are also found in plants and in various animal tissues **(Walsh and Wilcox, 1970).** Bacterial proteases are mostly extracellular, easily produced in larger amounts, thermostable and active at wider pH range. These properties make the bacterial proteases most suitable for wider industrial application **(Ward, 1985; Kalisz, 1988; Outtrup, 1990).**

On the basis of their acid-base behaviour, proteases are classified into three categories i.e. acid, neutral and alkaline proteases. The acid proteases are those which have pH optimum in the range of 2.0-5.0 and these are mainly fungal in origin. Proteases have optimum pH in the range of 7.0 or around are neutral and they are mainly originated from plants however some bacteria and fungi are also able to produce neutral proteases. While those which work in the pH range of 8.0-11.0 are alkaline proteases. Alkaline

proteases from the bacterial origin are the most important industrial enzymes, which contribute about 60% of the total world enzyme market **(Ward, 1985; Kalisz, 1988; Outtrup, 1990).**

Some of the important alkaline proteases are Solanain, Hurain and Proteolytic enzymes of Bacillus sp., Streptomyces sp. **(Hameed *et al*., 1996).**

These enzymes also have potential to contribute in the development of high value added products due to their characteristic nature of aided digestion. Among all proteases, alkaline proteases are robust in nature and are primarily used as detergent additives **(Prakasham *et al.,* 2006).** They are used in all types of laundry detergents and in automatic dishwashing detergents. Their function is to degrade proteinaceous stains. Moreover, they are also used for cleaning of membranes used in protein ultrafiltration **(Enshasy *et al*., 2008)**. They accounted for 40% of the total worldwide enzymes sales and 89% of the total protease sale and this trend is expected to increase in near future **(Ellaiah *et al*., 2002)**.

Bacteria are the most dominant group of alkaline protease. Bacillus being the most relatively prominent and serve as an ideal source of these enzymes biotechnological importance **(Gupta *et al*., 2002; Ramakrishna *et al*., 2010)** because of their rapid growth and limited space required for their cultivation (**Arulmani *et al*., 2007**).

The growth and enzyme production of the organism are strongly influenced by medium components like carbon and nitrogen sources. Besides the nutritional factors the cultural parameters like temperature, pH, incubation time were also plays a major role in enzyme production **(Jameel and Khan, 2011)** and as so the optimization of media components and cultural parameters is the primary task in a biological process.

Materials & Methodology:

Organism:

Bacterial strain B.subtilis was isolated from various region of Lucknow than screrned for enzyme production. The strain maintained on agar stants containing: **MD1(** Starch 2gm, Peptone 1gm, CaCO 3 0.3gm, Disstelled

water 100ml, pH=9), **MD2**(Starch 2gm, Peptone 1gm, Beef extract 1gm, $CaCO_3$ 0.3gm), **MD3**(Starch 2gm, Peptone 1gm, Yeast extract 1gm, $CaCO_3$ 0.3gm, Disstelled water 100ml, pH=9), **MD4**(Starch 2gm, Peptone 0.5gm, Beef extract 0.5gm, $CaCO_3$ 0.3gm, Disstelled water 100ml, pH=9), **MD5**(Nutrient-Gelatin 1gm, Glucose 1gm, KH_2PO_4 0.05gm, K_2HPO_4 0.05gm, $CaCl_2$ 0.05gm, Disstelled water 100ml, pH=9), **MD6**(Glucose 1gm, Peptone 0.5gm, Yeast extract 0.5gm, KH_2PO_4 0.1gm, $MgSO_4.7H_2$ 0.02gm, Na_2CO_3 0.5gm, Disstelled water 100ml, pH=9), **MD7**(maltose 1gm, Casein 0.5gm, Yeast extract 0.5gm, K_2HPO_4 0.1gm, $MgSO_4$ 0.2gm, Na_2CO_3 0.5gm, Disstelled water 100ml, pH=9) and **MD8**(Sucrose 1gm, Casein 0.5gm, Yeast extract 0.5gm, K_2HPO_4 0.1gm, $MgSO_4$ 0.2gm, Na_2CO_3 0.5gm, Disstelled water 100ml, pH= 9.

proteolytic activity assay

The alkaline protease activity was estimated by the procedure of modified Hagihara method using casein as substrate **(Hagihara *et al.*, 1958).** For this, the pure cultures obtained and identified were streaked on Alkaline Skim milk agar plate (Skim milk 1.0%, Peptone 0.1%, NaCl 0.5%, Agar 2.0% and pH 10.0). The Alkaline Protease production of the selected bacterial colony was confirmed by the formation of clear zones around the colonies. The isolate with maximum zone formation was maintained onto nutrient agar slant.

Submereged fermentation for alkaline protease production

Eight different enzyme production media were used for protease production. Culture suspension was inoculated in 250 ml of Erlenmeyer flask containing 100 ml culture medium. The medium was incubated for 48 h in shaker incubator at 37 °C. After 48 hours, the fermented broth was centrifuged at 10,000 rpm for 10 minutes to extract the crude extracellular Protease. The pellet which contains majorly the cell mass and debris was discarded and supernatant was collected. The supernatant was further used as crude enzyme extracts, used for enzyme assay and quantification.

Extraction and Purification of Protease

The crude enzyme extract was collected by above mentioned procedure and precipitated by continuous stirring at 4°C using varied percentages of ammonium sulphate solutions. The precipitated crude extract was harvested by centrifugation and dissolved in 0.1 M Sodium Phosphate buffer (pH 7.0). The precipitated enzyme was then subjected to Column chromatography with Silica Gel as the stationary phase, 0.1M phosphate buffer (pH 7.0) as Running buffer and 1M NaCl as elution buffer (pH 7.0) for further purification.

Protein concentration estimation

The protein concentration was determined by the Lowry method **(Lowry *et al.*, 1951)** using sodium carbonate, sodium hydroxide and Folin's Ciocalteau reagent. The colour change was measured at 600 nm using colorimeter. The protein concentration was estimated by comparing the value with standard graph prepared using Bovine serum albumin as standard protein.

Result & Discussion

The clear zone formation around the bacterial colony confirmed the proteolytic activity of *B. subtilis.*due to hydrolysis of casein. Hence the strains were identified as a protease producer and thus used for further experimental study.

Fig 1: A positive test of casien hydrolysis by the bacterial strain showing a clear zone around the streak indicating proteolytic activity of B.subtilis.

In eight different fermentation media. It was observed that MD8 produced highest amounts of alkaline protease which was ranged from (14.06 U/mL) in MD6 to (62.5 U/mL) in MD8 .

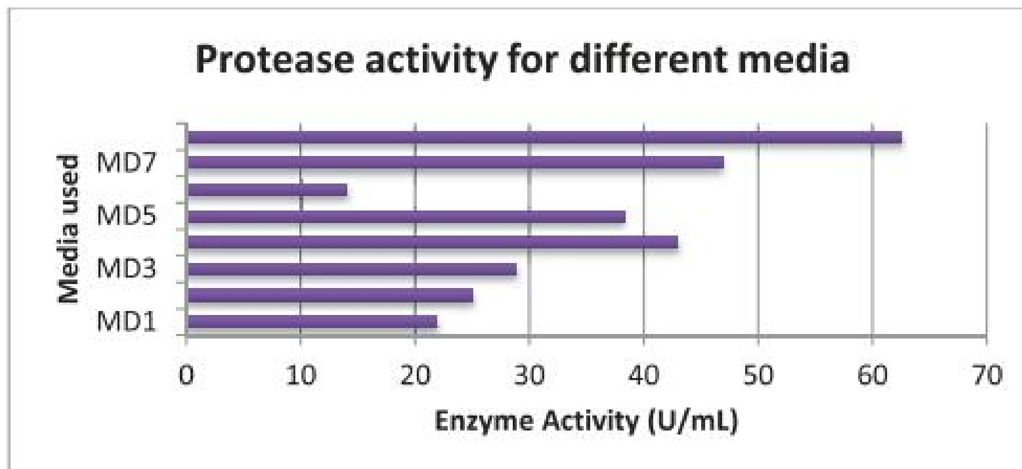

Fig 2: Protease activity for different fermentation media

The impact of media components and its environmental conditions play a major role in the final yield of enzyme during fermentation process. To obtain the most optimized conditions for media for Alkaline production by *Bacillus Subtilis* was thus determined using this study. pH, carbon, nitrogen sources and sodium chloride and metal ions on protease production was studied here.

Effect of pH on enzyme production

The optimum condition for the enzyme production by *B.subtilis* using MD8 media was found to be pH 11 (36 U/mL) as shown in Fig 4.

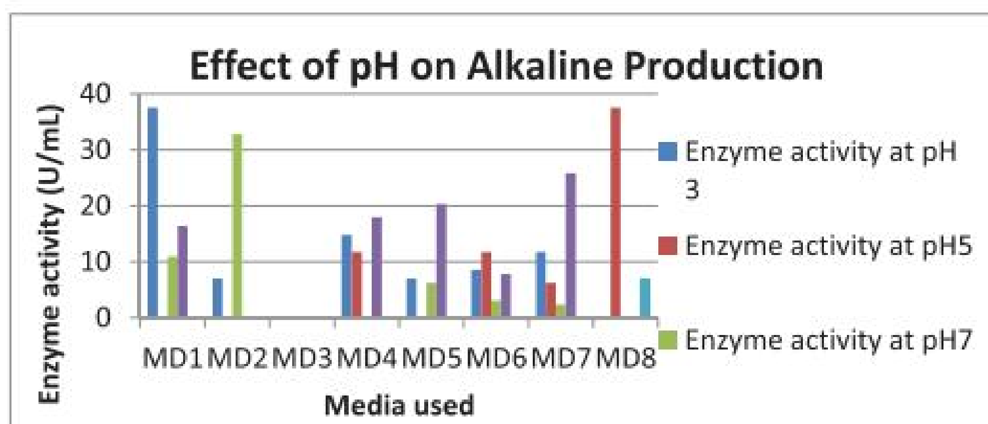

Fig 4: Graph depicting the Effect of pH on Alkaline Protease production by B. Subtilis

Effect of carbon sources of enzyme production

Glucose showed maximum yileld in MD2 (49.21 U/mL) and MD3 (41.4 U/mL) of protease followed by lactose in MD3 (33.59 U/mL) and MD7 (31.25 U/mL), Maltose in MD8 (21.87 U/Ml) as shown in fig 5. Media with sucrose as carbon source showed minimum yield in maximum number of enzyme production media. **(Gajju *et al.*, 1996).**

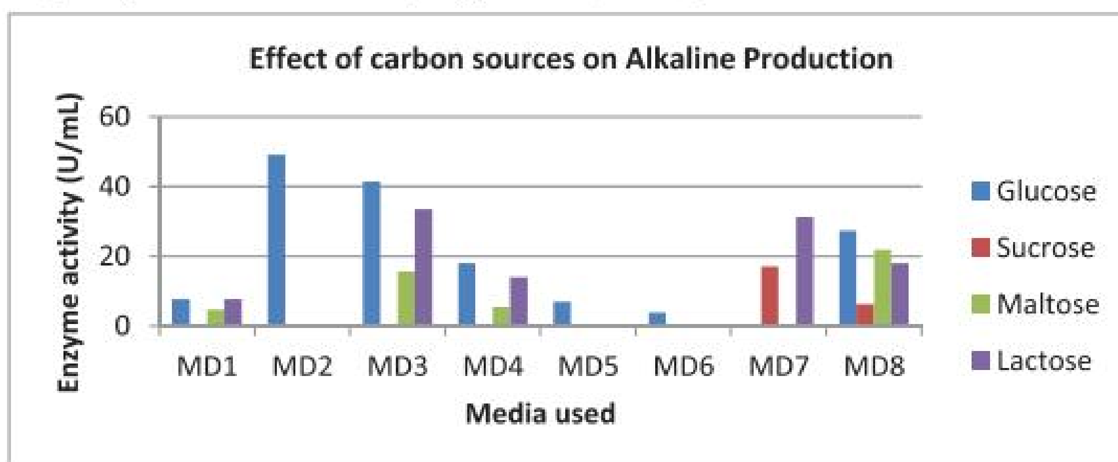

Fig 5: Graph depicting the Effect of different carbon sources on Alkaline Protease production by B. Subtilis

Effect of nitrogen sources on enzyme production

Among nitrogen sources, Ammonium chloride affected maximum (132.81 U/mL) protease activity for MD6 media followed by gelatin (109.37 U/mL), Urea (78.12 U/mL) and ammonium nitrate (70.31 U/mL) as shown in the fig 6**. (Rao *et al.*, 1998).**

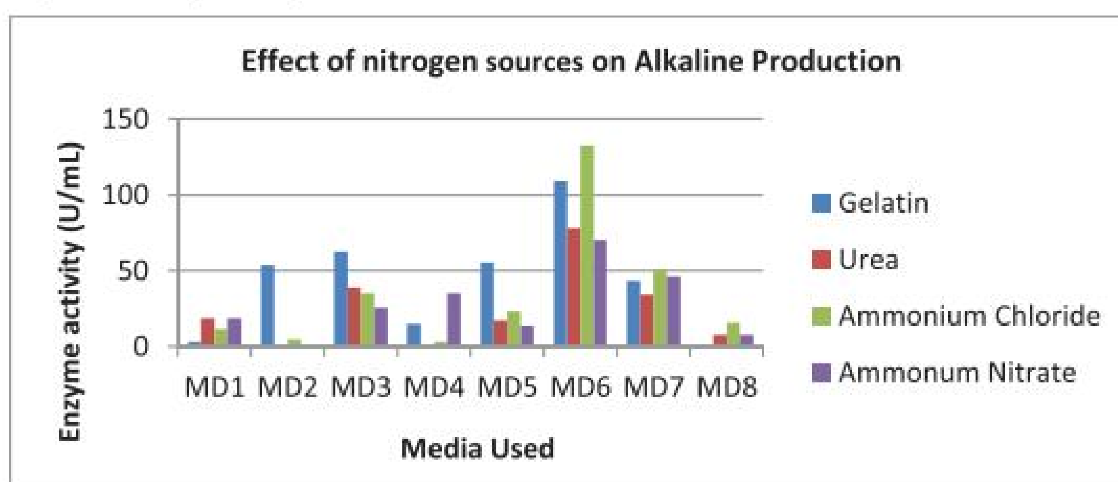

Fig 6: Graph depicting the Effect of different nitrogen sources on Alkaline Protease production by Bacillus Subtilis

Effect bof Nacl addition on enzyme production

In this study, effect of NaCl on the protease activity was determined by the enzyme activity of protease in presence of salt at different percentages (3%, 5%, 7%, 9% and 11%).It was found that maximum activity was seen in the case of MD5 with 11% NaCl (46.09 U/mL). Media supplemented with 5% NaCl also showed a significant activity of 31.25 U/mL and 38.28 U/mL for MD3 and MD5 respectively.

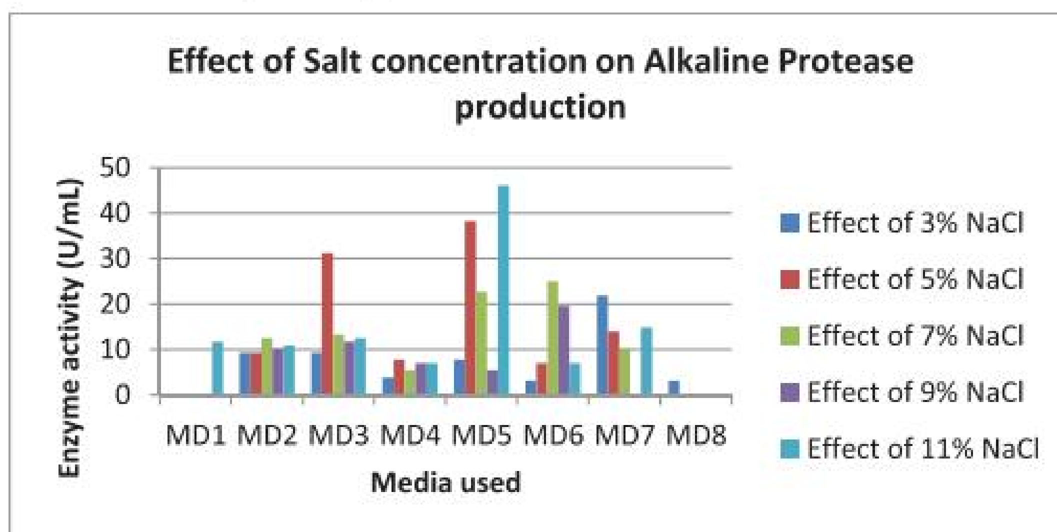

Fig 7: Graph depicting the Effect of different salt concentrations on Alkaline Protease production by Bacillus Subtilis

effect of metal ions on enzyme production

Metal ions's effect have been analysed on Alkaline protease production. From the result, it is evident that $MnCl_2$ (85.94 U/mL) plays better role in the alkaline protease activity followed by $MgCl_2$ (78.12 U/mL) in case of MD5 while $MgCl_2$ also showed a significant enzyme activity (70.31 U/mL) in MD4 media. $CaCl_2$ (26.56 U/mL) and KCl (30.46 U/mL) also showed a comparatively higher amount of enzyme activity for protease. Thus it could be inferred that media supplemented with any of these metal ions will help to elevate the activity of protease enzyme.

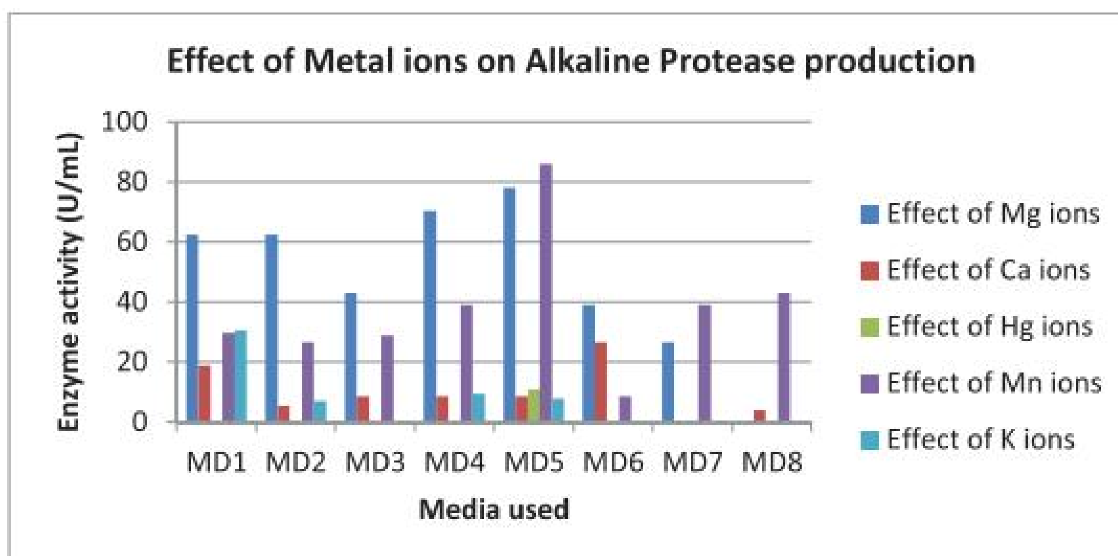

Fig 8: Graph depicting the Effect of different Metal ions on Alkaline Protease production by B. Subtilis

Conclusion

It is concluded from the present studies that *B. subtilis* was a good producer of alkaline protease in solid fermentation. Culture conditions such as, diluent pH, etc. had a profound effect on the production of enzyme. It was also found that suitable concentration of carbon and nitrogen sources resulted in significant rise in the protease production by *B. subtilis*

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