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Alkaline protease produced through Agro-Biproducts, applied in laundry cleaning

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Abstract: Alkaline protease is a major constitute of enzymatic group with nutritional and potential role in nature. Commercial success of alkaline protease have now started aiming at the engineering of novel enzymes that are more robust with respect to their pH, temperature, enzyme kinetics and molecular characterization techniques. New dimension of molecular diversity and technologies are used to improve performance characteristic by in vitro evolutionary changes. For the production of alkaline protease use of waste raw materials is cheaper and more advantageous than conventional substrates. In our studies agro-waste raw material such as gram powder, groundnut pod powder, mustard oil cake, feathers are used as substrates for the production of alkaline protease. All these substrates are cheap sources and most advantageous. Furthermore we will able to scale up the submerged state fermentation on gram powder. The thermal resistance, ability to function in a broad range of temperature may lead to conclude that future application of protease in laundry detergents formulations and in food and pharmaceutical industries is highly promising. Aklaline protease active at near pH 14.0 is produced with the use of agro- biproduct. Mustard oil cake found to be the best for the baterial isolate studied. The enzyme produced and partially purified is found to be very effective in cleaning laundry cloth.

Keywords: Alkaline protease, laundry cloth, submerged fermentation, purification, optimization.

I. INTRODUCTION

Alkaline proteases (EC.3.4.21-24) activity are those proteases which are active in a neutral to alkaline pH range 7-11 (Verrna et al., 2011). They have diverse applications in different industries such as detergent, food, feed, pharmaceutical, leather, silk and for recovery of silver from used X-ray films. In addition to above applications, alkaline proteases are also used to a lesser extent for other application such as contact lens cleaning, isolation of nucleic acid (Kyon et al., 1994), pest control (Kim et aL, 1999), degumming of silk (Puri et al., 20011). They are also used in the preparation of protein hydrolysates of high nutritional value, which play an important role in blood pressure regulation and are used in infant food formulation, specific therapeutic dietary products and the fortification of fruit juices and soft drinks (Ward et al.,1991), other application in food industry are used as crude preparation, in pharmaceutical industry they are used as ingredients of ointments for debridement of wards and in medicine preparation. Biotechnological importance of these enzymes has been realized by the leather industries for dehairing and bating hides as a substitute of toxic chemical. It account for about 60% of the total worldwide sales of enzyme. (Gupta et al., 2002) Since proteases are physiologically necessary for living organisms, they are ubiquitous, found in a wide diversity of sources such as plants, animals and microorganisms (Rao et al., 1998). Fortunately, enzymes can be separated from living cells and perform catalysis independent of their physiological environment. Commercial proteases are derived from animal tissues, plant cells and microbial cells via fermentation. But the inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases (Rao et al., 1998). Proteases of bacteria, fungi and viruses are increasingly studied due to its importance and subsequent applications in industry and biotechnology. Commercial application of microbial proteases is attractive due to the relative ease of large-scale production as compared to proteases from plant and animals. Microbial proteases, especially from Bacillus sp. have traditionally held the predominant share of the industrial enzyme market of the worldwide enzyme sales with major application in detergent formulations (Beg et al., 2003).

Enzyme cost is also the most critical factor limiting wide use of alkaline proteases for different applications. A large part of this cost is accounted for the production cost of the enzyme. Therefore, reduction in the production cost of enzymes could greatly reduce the cost of the enzyme. In submerged fermentation up to 40% of the total production cost of enzymes is due to the production the growth substrate (Enshasy et al., 2008). In this regard, uses cheap waste raw materials have enormous potential in reducing enzyme production cost. (Gizachew Haile,2009)

There are different agro waste substrates such as paddy straw, wheat bran, rice bran, rice husk, groundnut powder, cotton stalk , crushed maize which are used by different investigators (Vishawanata et al., 2010, Akcan et al., 2011). So Studies on alkaline protease that are produced from raw material in submerged state fermentation by microorganisms are scarce in literature. Keeping this in mind agrowaste raw materials such as gram powder, groundnut pod powder,

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mustard oil cake and feather are used for the production of alkaline protease throught bacterial isolates. The application study was done for its stain removing efficiency in laundry cloth.

II. MATRIAL AND METHOD

Isolation of Bacteria: Soil sampls were collected from protein rich area such as dairy industry, poultry farm etc. One gram soil was suspended in 10 ml of sterilized distilled water and serially diluted (10 to 10 -9) and Spread into Nutrient agar media. The Petri plates were incubated at 37°C for 24 hrs. Bacterial colonies were collected and maintained on lB medium.

Screening of protease producing bacteria: Skim milk agar plates were inoculated with the test cultures (Bacillus sp.). The plates were incubated at 37 °C for 24hrs. The zone of clearance around the colonies indicated the proteolytic activity of test cultures. This method was followed as as decribed by VidyaPallavi et al. (2011)

Quantitative assay of Alkaline protease: Fifty ml of assay medium (NB + 1% Casein) having pH 10 was inoculated with each isolate and incubated at 37°C for 48 hrs in shaker at 110 rpm. After incubation the broth cultures were subjected to centrifugation at 10,000 rpm for 10 minutes at 4°C in centrifuge. The supernatant obtained after centrifugation was used to determine the amount of the extracellular protease released into the assay medium.
The method described by Ahmad et al. (2010) was followed for protease. One mililitre of supernatant was taken in test tube and 5mL of alkaline copper reagent added to this. After 15 minutes 0.5mL Folin-ciocalteau reagent was added in each test tubes and stands for 30 minutes. The absorbance was read out at 700nm spectrophotometrically. One unit enzyme activity was defined as the amount of enzyme that releases li.tg of tyrosine per ml per min under the above assay conditions.

Protein estimation: The amount of protein was determined by the method of Lowry et al. (1951). Bovine serum albumin was used as standard. Culture filtrate was diluted 10 times and then 0.5 ml of diluted culture filtrate was taken in test tubes and 0.5 ml of distilled water were added in each tubes and 5m1 of Alkaline solution were added to the tubes. The tubes were kept at room temperature in dark for 10 min. After 10 min incubation 0.5 ml of Follin-Ciocalteau reagent were added to the tubes. The tubes were again incubated in dark for 30 min. Optical density was measured at 660nm using spectrophotometer.

Submerged state fermentation (SMF)

Inoculum Preparation: The inoculum was prepared by transferring a loopful of 24h old culture of Bacterial isolate into 100 mL of inoculum medium consisting of nutrient broth (pH10). The inoculated medium was incubated in a shaker for 24 h at 37 °C and 140 rpm for the propagation of bacterial growth up to 108-10 cells/ml. (Smita et al., 2012)

Production of alkaline protease: The method described by both Ahmad et al. (2010) and Akan et al. (2011) was modified slightly and used for the alkaline protease production. The initial growth medium was composed of peptone (0.5%), NaCI (0.05%), CaC12 (1%), MgSO4 (0.5%) and K2HPO4 (0.5%) and 10 gm of each agro-waste such as gram powder, groundnut pod powder, mustard oil cake and feather. Only gram powder was inoculated with sterilize water (p1-1-10) for control. It was inoculated with 1% (v/v) of 24h old inoculum broth. The pH of medium was adjusted at 10.0 pH with 1N HC1 / NaOH before sterilization at 121°C for 15 min. The inoculated medium was incubated in shaker at 37°C and 140 rpm for 24 h. Then the fermented broth was centrifuged at 9000xg for 10 min at 4°C to get clear supernatant containing enzyme solution.

Optimization of fermentation condition
All optimization studies carried in this study was based on the methods described by Akcan et al. (2011) with slight modification.

Effect of incubation time: To study the effect of incubation time on protease production, The basal medium along with different substrates such as groundnut pod powder, gram powder, mustard oil cake, feather was innoculated with the test culture islolates. Medium was maintained at pH10.0 and incubated at 37°C in shake. The alkaline protease activity was assayed at regular time intervals of 24,48,72,96,120,144, 192 hours.

Effect of different nitrogen source: To study the effect of nitrogen source such as skim milk, casein, tryptone, beef etract on production of enzyme the basal media was supplemented wirh thease. The production was assayed afer cultivation at 37°C in shaker to obtain growth.

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Effect of different pH: The effect of pH on alkaline protease production from the isolate was determined by growing the isolate in production media with different alkaline pH in the range of 10.0 to 14.0 using required concentrations of 1N NaOH.

Partial purification of alkaline protease
The partial purification of enzyme has been carried out as described by ammonium sulphate precipitation and dialysis.

Ammonium sulfate precipitation: Enzymes were precipitated with 65% ammonium sulfate and incubated overnight at 4°C. The suspension was centrifuged at 10,000 rpm for 15 minutes. The precipitate was collected and dissolved in minimum volume of 25 mM glycine-NaOH buffer (pH-10), Ammonium sulfate was found to activate the protease activity after dialysis.

Dialysis: Dialysis was carried out in semi permeable nitrocellulose membrane and dialized against 25 m M glycine-NaOH buffer.

SDS PAGE: Polyacrylamide gel electrophoresis (SDS-PAGE) was carried out at 10% (w/v) separating gel and 5% (w/v) concentration of stacking gel where f3-mercaptoethanol was used as reducing agent. Sample were loaded next to the standard protein marker. The gel was run at 30mA until the bromophenol blue reached the bottom of the gel. The gel was then transferred to staining solution for overnight then the distaining was done for 30 min. (Laemmli et al., 1970)

Application Studies

Stain removal studies: For stain removal study the method of Vijayalakshmi et al. (2011) was adopted. A clean piece of cloth was soaked in blood and other stains including coffee and tea. The cloth was then dried and soaked in 2% formaldehyde and washed with water to remove the excess formaldehyde. The partially purified protease was dropped on the cloth and incubated at 37°C for few minuits. After incubation, each piece of cloth was washed and dried. Sample without enzyme tratment was used as control

III. RESULT AND DISCUSSION

Several bacterial colonies form the dilution plates were picked up on the basis of their morphological differences. These colonies were subjected to the screening of protease production on skimmed milk plate (figure 1.). Out of twenty efficient proteolytic bacteria six isolates designated as p1, p2, p3, d4, 5m and 6m were selected for the quantitative assay of alkaline protease and grown on production broth. Here the isolate p4 produced highest (0.25U/ml) followed 0.22U/ml by d3 (figure 2). The isolate p4 was further used for the production of of protease through submerged fermentation of agro-biproducts (figure 3.). The best Similar study was also made by some other workers supporting the work such as Gessesse et al. (2003) isolated two alkaline protease producing alkaliphilic bacterial strain designated as AL-20 and AL-89 were isolated from a naturally occurring alkaline habitate. Katekan et al. (2009) isolated 171 strains out of them 169 bacterial isolates exhibiting proteaolytic activity and out of them two potential isolates namely TN51 and TN69 showing highest activity. Kalaiarasi et al (2009) isolated a protease producing bacteria from meat contaminated soil and identified as Pseudomonas fluorescens. Asokan et al (2010) isolated 6 bacterial strain from soil sample. Jameel et al. (2011) isolated 50 bacterial alkali Bacillus species from local habitate. Out of them 5 promising isolates were selected for production of enzyme. Smita et al. (2012) isolated 18 alkaline protease producing bacteria from water, soil and sediment sample.

The result revealed that the isolated grown on production mediun containing gram powder with D/w have produced maximun enzyme (figure 4). Ahmed et al. (2010) reported rice husk had maximum enzyme activity and protein concentration. Kanchana et al. (2010) observed that Bengal gram powder. Asokan et al. (2010) reported that starch have maximum enzyme activity. Vishwanatha. et al. (2010) reported that rice bran had maximum enzyme activity. Akcan et al. (2011) observed that lentil husk had maximum enzyme activity.

The study revealed the maximum protease production at 144 hrs of submerged fermentation time (figure 5.). Ahmed et al. (2010) reported that 48 hrs fermentation time period stimulates maximum enzyme activity. Kalaiarasi et al. (2009) observed that 24 hr ha maximum enzyme activity. Akcan et al. (2011) described that at 48 hrs incubation time had maximum enzyme activity. Smita et al. (2012) reported that 120 hrs. incubation time had, maximum enzyme activity.
The presence of tryptone as nitrogen supplement in production media is found to be maximum effect on enzyme activity and maximum protein concentration among all nitrogen sources (figure 6) . Ahmed et al. (2010) reported skim milk as a nitrogen source has a significant effect on protease production and shows maximum enzyme activity, total

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protein. Kalaiarasi et al. (2009) reported that peptone stimulate protease activity. Akcan et al. (2011) observed that beef extract was found to be the best inducer of alkaline protease, while other nitrogen sources repressed enzyme production. At pH 14 had maximum protease activity (figure 7) which significantly decreased with lower pH value. This is the best output of the study. Usharani et al. (2010) reported that pH 7 had maximum enzyme activity. Kalaiarasi et al. (2009) observed that pH 9 had maximum enzyme activity. Ahmed et al. (2010) described that pH 11 gave the highest protease activity. Kanchana et al. (2010) reported that pH 10 stimulate protease production. Smita et al. (2012) described that pH 10 had maximum enzyme activity.

The partially purified alkaline protease was applied on the cloth stained with blood and tea water for laundry cleaning (figure 8). The enzyme showed very effective for destaining the cloth with blood. The cloth stained with the tea water was destained less effectively. The presence of protein in the stain proteolyse with the tresence of the protease enzyme. Being an alkaline protease this can be effectively used for laundry detregent formulations.

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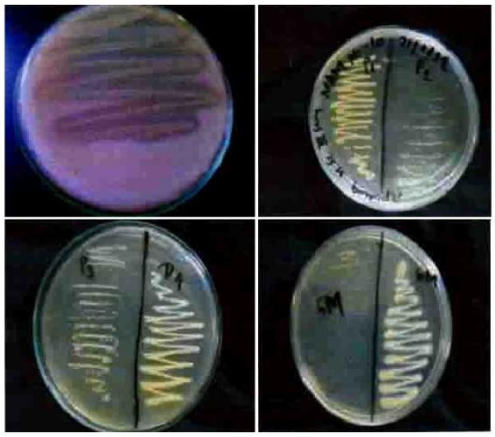

Figure 1. Screening of Bacterial isolates

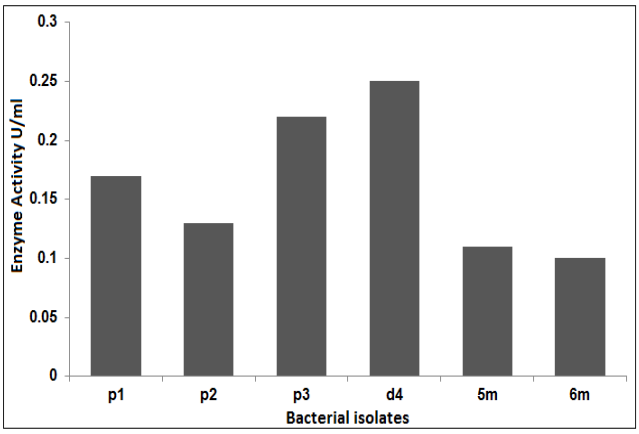

Figure 2. : Enzyme production of different isolates

Figure 3. Sustrates used for enzyme production

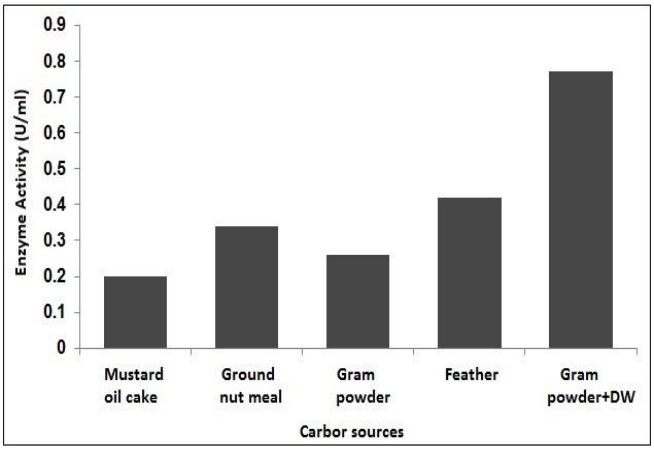

Figure 4. Enzyme production through different substrate

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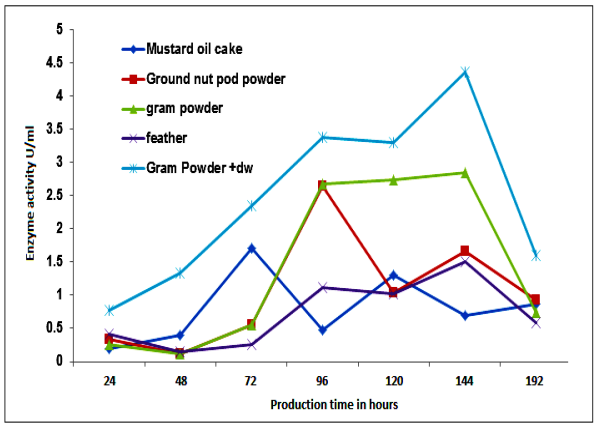

Fig5. Time optimization of enzyme production

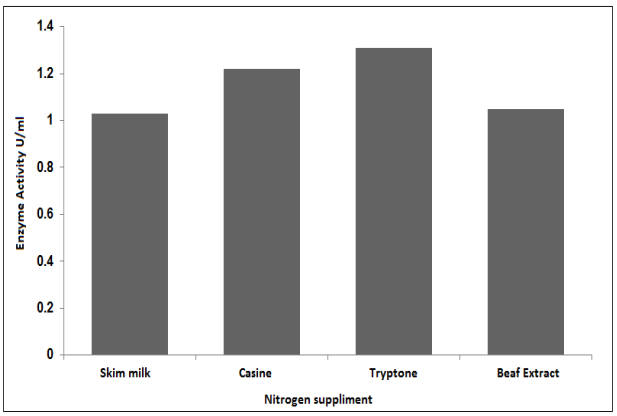

Fig 6. Nitrogen suppliment optimization for production enzyme

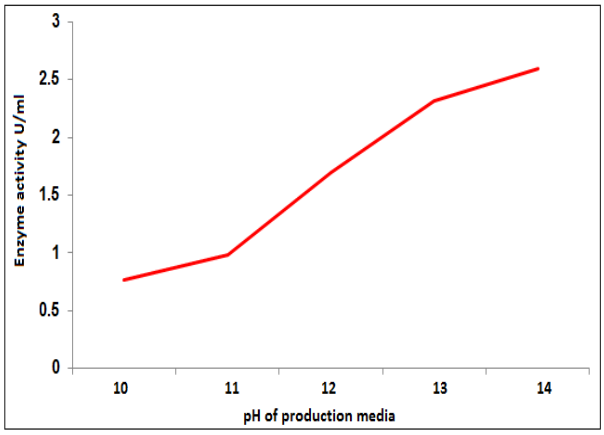

Figure 7. pH optimization for enzyme production

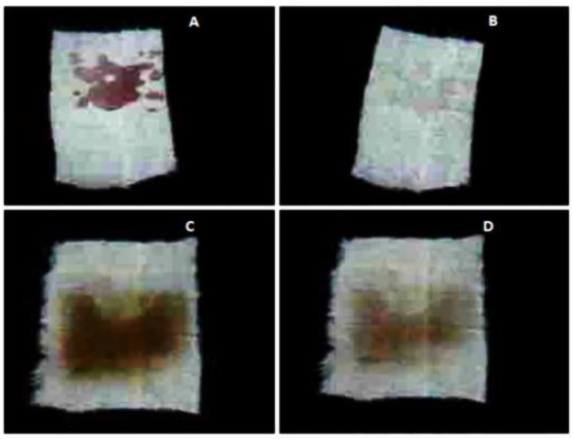

Figure 8. Stain removal from laundry cloth.

A. Blood stained cloth, control
B. Blood stained cloth, treated with enzyme
C. Tea stained cloth, control
D. Tea stained cloth, treated with enzyme