RECENT ADVANCES IN MICROBIAL PRODUCTION OF PROTEASES

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ABSTRACT: *Industrial enzymes are the key to success of bioprocesses. New and emerging applications of these enzymes have opened new vistas in field of their production methods immensely. The industrial technical enzymes used for detergent, pulp and paper manufacturing, have a largest segment with nearly half of market share. Amongst these enzymes the largest share has been held by alkaline proteases, hence being the most valuable commercial enzyme. Alkaline proteases find their applications in the detergent and leather industries and there is an ever increasing trend to develop eco-friendly technologies. The microbes are best source for protease production because of their rapid growth and the ease with which they can be genetically manipulated to produce new enzymes with desirably changed properties according to the need of respective industry. Pseudomonas is a gram-negative bacterium that predominantly produces alkaline proteolytic enzymes. Fungal alkaline proteases are advantageous because of the ease of downstream processing to prepare a microbe-free enzyme economically. In future studies efforts can be made for upgrading the protease production technology from laboratory to a large-scale process viz. enzymatic treatment of protein fibers, like hair, wool and silk. Development of new formulations for industrial and domestic wool carpet cleaning, garment washing, dyeing processes for protein fibers by pre-treatment with the proteases can be performed. The present review elaborates the microbial protease production technologies and also studies their applications in various industries.*

I. INTRODUCTION

Biotechnology is gaining ground rapidly due to the various advantages that it offers over conventional chemical processes especially regarding environment and cost involvement. Industrial enzymes represent the heart of biotechnology processes. The global market for industrial enzymes increased from $2.2 billion in 2006 to $2.3 billion by the end of 2007, $2.7 billion by 2012 and $4.9 billion by 2013 (BCC-Business Communications Company, Inc., 2008, 2013). New and emerging applications have helped drive demand for enzymes and the industry is responding with a continuous stream of innovative products (Chander and Kaur, 2015). The industrial enzyme market is divided into three application segments: technical enzymes, food enzymes and animal feed enzymes. Technical enzymes for detergent, pulp and paper manufacturing (Chander and Arora, 2014), have a largest segment with approximately 52% dominated by alkaline proteases.Proteases cover the 60% of total enzyme market and amongst the most valuable commercial enzyme. Alkaline proteases hold a great potential for application in the detergent and leather industries and there is an ever increasing trend to develop environment friendly technologies (Oberoi et al., 2001; Yang et al., 2006). Plants, animals and microbes are the main sources for protease production. The preferred sources of proteases are microbes because of their rapid growth and the ease with which they can be genetically manipulated to generate new enzymes with altered properties and are currently being utilized by the detergent industry e.g. Serine proteases produced by Bacillus strains (Harwood and Cranenburgh, 2008). Proteases from several bacteria have been purified and characterized. Genus Pseudomonas a gram-negative bacterium that predominantly produces alkaline proteolytic enzymes and the proteases has been purified. Fungal alkaline proteases are advantageous for their easy downstream processing, low cost.

The bulk of the plant proteases e.g. Papain, Bromelain and Ficin have major application in the food industry where they are added at different stages of production. Thermo stable serine proteases named 'wrightin' from the latex of the plant *Wrightia tinctoria*, 'Carnein' from the latex of the weed *Ipomoea carnea* sp. fistulosa (Morning glory) and 'Milin' from the latex of *Euphorbia milii*, too have found applications in food and other biotechnology industries (Nieri *et al.,* 1998).

A 70kDa serine protease was identified from artificially senescing parsley leaves this protease activity is low in young leaves, was found to increase considerably in parallel to the advance of senescence and the reduction in the protein content of the leaves (Jiang *et al.,* 1999; Hortensteiner et *al.,* 2002).

However, till date there are no reports of utilization of protease in industry from this abundant senesced leaf waste.

The innovative aspect of the present review is to identify and study details of alkaline proteases from various biological sources such as senesced leaves of regional plants that are currently not used in agriculture and from soil microbes with a purpose to have positive effect for solid waste management. The purified enzyme may found potential industrial application. The first objective of the present study is to illustrate new sources of alkaline proteases e.g.:

i. From senescing leaves as it shows dominance of proteolytic enzymes.
ii. Soil sample of the poultry waste site which is rich in organic waste was selected for Screening of microbial isolate that can produce alkaline protease.

Secondly, check for application of isolated enzymes in industry processes. Since there are no reports available on the use of plant proteases in detergent industry we were interested in exploiting the use of plant proteases for its commercial application in detergent industry. The leaves of *Lantana camara* after initial screening for protease from various plants. It is the most commonly
occurring weed in the world and the proteases extracted from this weed would be cost effective. We have reviewed an easy protocol of production and potential application of caseinolytic, thermo stable alkaline protease by utilizing the senesced leaves of common weed L. camara and a novel extracellular alkaline protease of soil bacterium, Pseudomonas thermaerum GW1. Protease from senesced leaves of the weed Lantana camara can purified by a two-step procedure involving ammonium sulfate precipitation and Sephadex G-250 gel permeation chromatography. The Sephadex G-250 fraction of senesced leaves of Lantana camara showed 28.31 fold with a yield of 6.19%. The enzyme was shown to have a low molecular weight of 43 kDa by SDS-PAGE. It was strongly activated by metal ions such as Cu2+, Zn2+, Mg2, Co2+ and Mn2+. It remained active at 60°C, pH 10.5 even after 1 hour of incubation when casein was used as substrate. The compatibility of the enzyme when studied with commercial and local detergents, 60% activity of the enzyme was retained even after 1 h of incubation at pH 10.0. The easy availability of the senesced leaves of this common weed makes it a cheaper enzyme source and potential additive in detergents (Gaur et al., 2008)

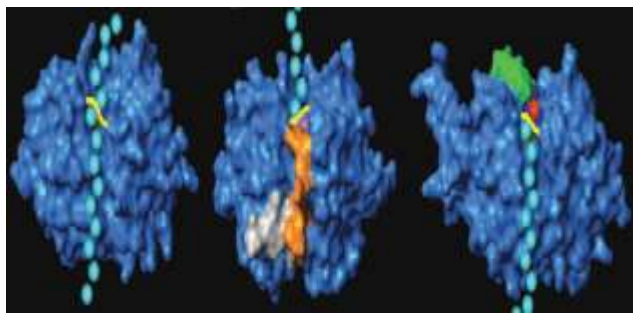
An extracellular protease was purified and characterized from Pseudomonas thermaerum GW1 a new strain identified isolated from soil of Poultry waste site by Gaur et al., (2009). The strain produced extracellular protease in the culture media that was maintained at 37°C and at 140 rpm. The media was harvested for protease after 48 hrs of incubation at 37°C in basal media supplemented with 1% casein. Enzyme was purified by ammonium sulphate precipitation and DEAE-cellulose chromatography. The molecular weight of the enzyme was estimated to be 43kDa as shown by casein zymography studies. The optimum pH for the proteolytic activity was pH 8.0 and enzyme remained stable between pH 5 -11 at 60°C. Interestingly Mn2+ (5mM) strongly activated enzyme activity by 5 fold, while Cu2+, Mg2+and Ca2+ moderately activated enzyme activity, whereas Zn2+, Fe2+ and Hg2+ inhibited enzyme activity. The protease produced was stable in presence of 50 % (v/v) ethyl acetate and acetone and however showed 50% reduction on enzyme activity in the presence of glycerol. (Gaur et al., 2009).

Future studies regarding upgrading the protease production technology from laboratory to a large-scale process, allowing for a new green industrial process to be developed especially where enzymatic treatment of protein fibers, like hair, wool and silk is involved so that there is significant reduction in the chemical use and cost. Development of new formulations for industrial and domestic wool carpet cleaning, garment washing, dyeing processes for protein fibers by pre-treatment with the proteases can be performed. The amino acid sequence determination of purified alkaline protease from senesced leaves of Lantana camara and P.thermaerum would be performed and checked for innovative application in other biotechnology industries.

II. CLASSIFICATION OF MICROBIAL PROTEASES

According to the Enzyme Commission (EC) classification, proteases are members of the group 3 (Hydrolases), and sub-group 4 (hydrolyzing peptide bonds). Proteases have been divided into two broad groups on the basis of their ability to hydrolyze N- or C- terminal peptide bonds (exopeptidases) or internal peptide bonds (endopeptidases). The exopeptidases are also used in some commercial applications, endopeptidases are industrially more important than the former. Exopeptidases are subdivided as aminopeptidases that cleave the N-terminal peptide linkage and carboxypeptidases that cleave the C-terminal peptide bond. Several other features have also been used in classifying proteases into different groups such as occurrence of charged moieties at sites relative to susceptible bond , their pH optima (as

acidic, neutral or alkaline), substrate specificity (collagenase, keratinase, elastase), or their homology to previously characterized proteases such as trypsin, pepsin and others (trypsin-like, pepsin-like). Morihara (1999) classified serine proteases as trypsin-like proteinases, alkaline proteinases, Myxobacter lytic proteinases and Staphylococcal proteinases. Hartley (2000) classified endoproteases into four groups on the basis of their active site and sensitivity to various inhibitors.



(a) Endopeptidases (b) Aminopeptidases (c) Carboxypeptidases

A. Serine Proteases

Serine proteases have a serine group at their active site. They are abundant and wide spread among viruses, bacteria and eukaryotes, suggesting their vital importance to organisms. Serine proteases are inactivated by organic phosphate esters which acylate the active serine residue. Serine proteases are generally active at neutral and alkaline pH, having an optima between 7 and 11.

B. Cysteine/Thiol Proteases

Cysteine proteases are found both in prokaryotes and eukaryotes. They have cysteine at their active site and have pH optima in the range of 6 to 8. They are activated by reducing agents such as hydrogen cyanide and inhibited by oxidizing agents. They are susceptible to sulfhydral (-SH) agent such as p-CMB but are unaffected by DFP and metal-chelating agents. The activation by reducing agents is due to regeneration of SH group. They have temperature optima between 50- 70°C.

C. Aspartic Proteases

These proteases are widely distributed in animal, yeast and mould cells but rarely found in bacteria. They are commonly known as acidic proteases having aspartic acid residues at their active sites. They show specificity towards aromatic or bulky amino acids residues on both sides of the peptide bond and have pH optima 5 between 3-4. The aspartic proteases are inhibited by pepstatin. Microbial aspartic proteases are further divided into two groups, produced by moulds and yeasts.

(a) Pepsin like proteases and
(b) Rennin-like protease

D. Metalloproteases

Metalloproteases are the most diverse of the catalytic types of proteases. They are characterized by the requirement for a divalent ion for their activity. These enzymes are sensitive to chelating agents but insensitive to sulfhydral agents and phosphate esters have metal ion involved in the catalytic mechanism. Neutral and alkaline proteases from many microbial sources belong to this group of enzymes.

III. PRODUCTION OF PROTEASE FROM BACTERIA

Most commercial proteases (neutral and alkaline) are produced by organisms belonging to genera Bacillus. Bacterial neutral proteases are active in a narrow pH range i.e., pH 5-8 and have low heat tolerance. Due to their intermediate rate of reaction, neutral proteases generate less bitterness in hydrolyzed food proteins than the animal proteases so are valuable for application in the food industry.

Neutrase, a neutral plant protease is insensitive to the inhibitors and is therefore useful in the brewing industry. The bacterial neutral proteases are characterized by their high affinity for hydrophobic amino acid pairs. Their low thermo tolerance is advantageous for controlling their reactivity during the production of food hydrolysates with a low degree of hydrolysis. Some of the neutral proteases belong to the metalloproteases type and require divalent metal ions for their activity, while others are serine proteases, which are not affected by chelating agents.

Bacterial alkaline proteases are marked by their high activity at alkaline pH for e.g. pH 10 and their broad substrate specificity. Their optimal temperature is around 60°C. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry.

3.1. Production from Pseudomonas sp.

Partial purification and characterization of alkalophilic protease production from Pseudomonas aeruginosa was isolated from the gut of marine and coastal waters shrimp Penaeus monodon (Kumar et al., 2011). The protease production was assayed in submerged fermentation to produce maximum protease activity (423 ± 0.09 U/ml). The enzyme was precipitated with ammonium sulphate and partially purified by ion exchange chromatography through DEAE Sephadex A-50 column. In 10th fraction showed maximum protease activity (734 ± 0.18 U/ml) with increase in purification fold. The molecular weight of protease from P. aeruginosa was recorded as 60 kDa. The stability of protease was tested at various pH and temperature; it showed maximum protease activity at pH-9 and temperature 50ºC. Among the various surfactants tested for enzyme stability, maximum activity was retained in poly ethylene glycol. The compatibility of protease enzyme with various commercial detergents; the enzyme retained maximum protease activity in tide. The results are indicated that all these properties make the bacterial proteases are most suitable for wide industrial applications.

3.1.1. Methodology

(a) Protease producing organism

The organism P. aeruginosa originated from the gut of Penaeus monodon marine coastal area in Kanyakumari District, Tamilnadu. Hence, an attempt was made by the authors to isolate and characterize protease producing bacteria from marine shrimp; the gut was dissected out from 4 or 5 shrimps in aseptic condition. The dilutions from gut were spread on the Zobell marine agar and colonies with different morphology were selected and purified in nutrient agar plates (Kumar et al., 2011).

(b) Effect of incubation period on enzyme production

The effect of incubation period on protease production was tested by the team, P. aeruginosa inoculated on casein broth medium and incubated at 37°C. The culture growth was determined by read at 600 nm in UV-VIS Spectrometer and enzyme activity was estimated for every 6 hrs intervals until to reach a decline phase (Kumar et al., 2002).

(c) Mass scale enzyme production

The protease producing organism P. aeruginosa was cultured by the authors in the production medium. The medium containing (g/l): casein-8g; glucose-10g; beef extract-5g; CaCl2-0.1g; NaCl-30g; pH-9 in 1000 ml Erlenmeyer flask and inoculated with 2% inoculum of P. aeruginosa and incubated at 37°C for 72 hrs. The flask was kept in a shaker at 240 rpm. The cells were then harvested by centrifugation at 10,000 rpm for 15 min at 4°C to obtain supernatant was collected (Banerjee and Ray, 2006).

(d) Ammonium sulphate precipitation

The enzyme was precipitated by addition of ammonium sulphate at a level of 60% saturation and the precipitate was stored at 4°C for until further analysis and purification by Ion exchange Chromatography using DEAE Sephadex (Jewell and Falkinham, 2000).

3.1.2. Characterization of protease

(a) Effect of pH on protease activity

The effect of pH on protease activity was examined at various pH levels (pH 4-10). The optimum pH for protease activity was studied by the enzyme was pre incubated with various pH buffers such as acetate buffer (4, 5), citrate phosphate buffer (pH 6), Tris-HCl buffer (pH 7-8) and carbonate buffer (pH 9-10) at 37°C for 30 min. The residual activity of protease was estimated under standard assay conditions (Doddapaneni et al., 2007).

Fig. 1: Zymogram analysis of DEAE Sephadex A-50 purified fractions of protease Lane 3, 4: Showing purified fractions of protease hydrolyze the gelatin to form clear zone

(b) Effect of temperature on protease activity

The optimum temperature for protease activity was studied by the enzyme was pre-incubated with carbonate buffer (pH-10) at different temperatures ranging from 30 to 90°C for 30 min. The residual activity of protease was estimated under standard assay conditions (Doddapaneni et al., 2007).

(c) Effect of surfactants on protease activity

The protease enzyme was pre-incubated with Triton X-100, Sodium dodecyl sulphate (SDS), Poly ethylene glycol (PEG), Tween-20 and Tween-80. The enzyme was incubated without any surfactant was taken as control. The residual activity of protease enzyme was estimated under standard assay conditions (Doddapaneni et al., 2007).

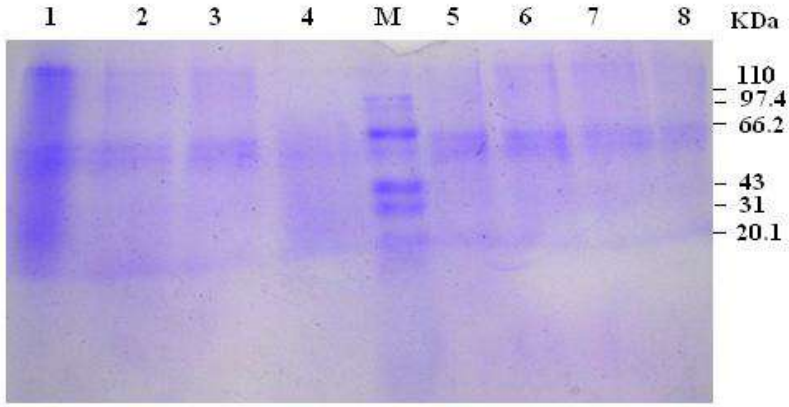


Fig. 2. SDS-PAGE Profile of Protease enzyme (60 kDa) Lane 1: Showing crude extract of protease,Lane 2-8: DEAE Sephadex A-50 purified fractions of protease, Lane M: Standard Protein Molecular Weight Marker.

3.2. Production from Bacillus sp.

The microbial alkaline protease production by using isolated Bacillus subtilis has been exercised industrially. B. subtilis which produces an extracellular alkaline protease was isolated from meet surface purchased from local Egyptian markets (Eftekhar et al., 2003). Maximum enzyme activity was achieved when the bacterium was grown on corn steep liquor (2.0 %, w/v) instead of soybean meal (2 %) followed by casein hydrolysate (2%, w/v) and 12 % cane sugar molasses as a carbon source at pH 10.0 and 37ºC over 24 hrs incubation period (maximum enzyme production at 48 hrs). The enzyme has an optimum pH of around 10.0 and maintained its stability over a broad pH range between 5.0 to 12.0. Its optimum temperature is around 37 °C, and exhibited a stability of up to 50 ºC.

3.2.1. Material and Methods

(a) Microorganism and culture maintenance

The bacteria was isolated from surface of meet samples, screened using a nutrient agar plates and later in alkaline broth. Stock cultures of the isolate were stored in 20% glycerol at −70 °C. Prior to each experiment, the bacterium was subcultured from the frozen stocks onto alkaline agar (pH 10.5) containing (g l−1): glucose, 10; peptone, 5; yeast extract, 5; KH_2PO_4, 1; $MgSO_4 \bullet 7H_2O$, 0.2; Na_2CO_3, 10 and agar, 15. Alkaline broth was used as the basal medium for preliminary studies of the bacterial growth and protease production.

(b) Gelatin Clearing Zone Technique

The protease enzyme activity was determined by El-Safey and Ammar, (2002) briefly, according to gelatin clearing zone (GCZ) technique. In this assay, soluble gelatin (1 % w/v) was emulsified and supplemented with (1.5 % w/v) Bacto-agar, pH was adjusted as required with proper buffer (e.g. phosphate buffer at pH 7.0) cups were made (3 cups optimal) in each plate. Equal amounts (0.1 ml suitable) of extracted enzyme (or enzyme solution) to be assayed were introduced into each cup. The plates were incubated at 35 ºC for 24 hrs., at the end of incubation time, the plates were flooded with previously prepared Mercuric chloride (HgCl) in HCl solution (HgCl, 15g and 20 ml of 6N HCl completed to 100 ml with distilled water), and the mean diameters of recorded clearing zones were calculated.

(c) Growth Medium

The alkaline medium (pH 10.0) used for protease production contained (g %)(w/v): glucose, 6; soybean meal, 2.0, calcium chloride, 0.04, magnesium chloride, 0.02, sodium dihydrogen phosphate, 0.09 and disodium hydrogen phosphates, 0.62. After autoclaving and cooling the medium, 10 ml of a trace element solution containing (g l−1): $Na_3C_6H_5O_7$, 10; $(NH_4)_6MO_7O_{24}$, 0.1; $FeSO_4 \bullet 7H_2O$, 2; $CuSO_4 \bullet 5H_2O$, 0.2; $ZnCl_2$, 0.2, was added to one liter of the medium. The operating conditions were maintained at a temperature of 37°C and agitation of 150 rpm on the shaking incubator for 48 hrs.

(d) Protease Assay

The proteolytic activity of the enzyme was quantitatively measured by a modified method of Joo et al., 2002. Substrate solution was consisted of 1% casein in 0.1M Tris–HCl buffer at pH 9.0. The assay mixture consisted of 450 µl of substrate solution and 50 µl of enzyme solution (culture supernatant) suitably diluted with 0.1M Tris–HCl buffer (pH 9.0). The mixture was incubated at 35°C for 10 min and the reaction was terminated by adding 500 µl of 10% trichloro acetic acid (TCA) followed by centrifugation at 5000×g for 15 min to remove the precipitate. Protease activity was determined as the amount of released tyrosine from the supernatants at 275nm. One unit of enzyme activity was defined as the amount of the enzyme resulting in the release of 1 µg of tyrosine per min per ml at 37°C under the reaction conditions.

(e) Different Factors Affecting Protease Production by the Bacterial Isolates

The study was carried out at 250 ml conical flask each containing 25 ml growth medium. The flasks were inoculated by 0.5 ml of inoculum prepared and incubated at specific temperature in a rotary shaker (180 rpm). At the end of fermentation period, the culture medium was centrifuged to obtain the culture filtrate that was used as the enzyme source. In all experiments, the biomass dry weight, final pH and protease production were monitored (Joo et al., 2002).

3.3 Production from Micrococcus sp.

The protease producing bacterial isolate and identified as *Micrococcus luteus (*Amara *et al.,* 2009). The optimum conditions observed for protease production was 37°C at pH 7, with 1% inoculum in the medium for 24 hrs of incubation in *M. luteus* while in *Bacillus* species, the optimum conditions observed was 47°C at pH-9, with 2% inoculum concentration in the medium for 96 hrs of incubation. Generally, temperature and pH had more effect on the protease activity of *M. luteus* while inoculum concentration and time of incubation had more effect on the protease activity of *Bacillus* species*.* The study gave evidence that these bacterial isolates could be potentially applied in biotechnological processes.

3.3.1. Material and Methods

(a) Sample Collection and Isolation of Proteolytic Bacteria

The soil samples were collected from an abattoir environment in Owerri, Imo State, Nigeria. They were stored in ice and analyzed within one hour of collection. One gram of soil sample in a 250 ml flask was homogenized with 10 ml of sterile water; it was later made up to 100 ml with sterile water, mixed and shaken on a mechanical shaker for 45 min. An aliquot of the homogenized sample (0.2 ml) was spread on casein agar plates (nutrient agar supplemented with 36% casein) and incubated for 48 hrs at 37°C. The isolates were identified based on their morphological and biochemical characteristics.

(b) Screening of Proteolytic Bacteria

The screening method described by Amara et al., (2009) was adopted. Briefly, to 100 ml of sterile water contained in a 250 ml flask was added 3 g of skim milk and autoclaved for 15 min. after sterilization, the suspension was decanted and the soluble solution was added to sterile water agar (16 g agar/l). The mixture was gently stirred until completely homogenized and then distributed in petri dishes. After media solidification, the bacterial cultures were inoculated on the plated and incubated at 37°C. Clear zones around the bacterial colonies indicate the presence of proteolytic activity which can be confirmed using coomassie blue staining method.

(c) Coomassie Staining Method

This was performed using the method of Weber and Osborn. Coomassie blue was dissolved in a solution of methanol-acetic acid-water (5:1:4 v/v/v) to achieve 0.25% (w/v). Ten milliliter of the staining solution was added to each of the plates and incubated at room temperature for 15 min. After the incubation the staining solution was removed from the plate and the plate gently washed with distilled water. Thereafter, the plates were de-stained with a solution containing 66 ml methanol, 20 ml acetic acid and 114 ml of distilled water till there was a clear contrast between the plate's background and the degradation zones around the bacterial colonies.

(d) Effect of Temperature on Protease Production

The effect of temperature on protease production was studied by incubating the culture media at different temperatures ranging from 27°C-77°C for 24hrs. Protease activity was determined after 24hrs of incubation (Kalaiarasi, and Sunitha, 2009).

(e) Effect of pH on Protease Production

The effect pH on protease production was determined by culturing the bacterium in the protease production media with different pH ranges (pH 5 to 11). The enzyme assay was carried out after 24hrs of incubation at 37°C (Kumar et al., 2011).

IV. PRODUCTION OF PROTEASE FROM FUNGI

Fungi elaborate a wider variety of enzymes than the bacteria for example Aspergillus oryzae produces acid, neutral and alkaline proteases. The fungal proteases are active over a wide range of pH up to 4 to 11 and exhibit broad substrate specificity. However, they have a lower reaction rate and worse heat tolerance than the bacterial enzymes. Fungal enzymes can be conveniently produced in a solid state fermentation process. Fungal acid proteases have an optimal pH 4 and 4.5 and are stable between pH 2.6 and 6.0. They are particularly useful in cheese making industry due to their narrow pH and temperature specificities. Fungal neutral proteases are metalloproteases that are active at pH 7.0 and are inhibited by chelating agents. Due to their peptidase activity and their specific function in hydrolyzing hydrophobic amino acid bonds, fungal neutral proteases supplement the action of plant, animal and bacterial proteases in reducing the bitterness of food protein by hydrolysates. Fungal alkaline proteases are also used in food protein modifications.

4.1. Production from Aspergillus sp.

There are various sources of proteases as they occur naturally in all organisms and constitute 1 to 55% of the gene content depending upon the more of attach they have been broadly classified as exoproteases and endoproteases French beans are commonly grown in India and this fairly rich in protein content as well as minerals (Ito et al., 1998). French bean meals thus can be modified suitably for the growth of fungal species. Their study supports the fact that French beans are a moderate type of solid state fermentation substrate for protease production using Aspergillus sp. The aim of this study was Isolation and identification of protease producing Aspergillus sp, solid state fermentation of French bean meal using isolated Aspergillus sp. French bean meal fermentation medium as a solid state system reveals encouraging results.

4.1.1. Material and Method

(a) Sample Collection and Isolation of Aspergillus sp.

Various vegetables and fruits were collected from the market showing spoilage to the extent that the integuments and the outer skin damaged extensively. The sample was examined microscopically after staining fungal infected samples were screened and scrapings were inoculated on Potato Dextrose Agar (PDA) and grown at room temperature for 7 days. The PDA plates showing intense mycelia growth with profuse brown to black spores were selected and repeatedly purified over PDA to get axenic cultures (Bhalla et al., 1999).

(b) Characterization and Confirmation of Isolates

The axenic fungal culture showing brown to black spores were subjected to Microscopic examination by lacto phenol cotton blue staining. A wet mount was prepared on the slide with the culture from the PAD plates the mount was stained with a few drops of cotton blue and covered with a cover slip. The typical morphology of Aspergillus was observed under microscope (Cappuccino and Sherman, 2004).

The species of Aspergillus confirmed by subjecting it to standard slide culture technique. A thick layer of PDA was laid over the slide aseptically and then they were inoculated with the fungal isolates. The slides were put in sterile Petri plates and incubated at room temperature for development of vertical hyphae, microscopic examination of the slides were done to confirm Aspergillus sp.

(c) Solid State Fermentation of French Bean and Estimation of Protease Activity Media was developed with some modifications including dehulled seeds were converted into a semisolid pest using a varying grinder. The resulted pest was mixed with phosphate buffer and mineral solution. Protease was estimated by Folin's reagent using tyrosine as standard as per the method described in standard methods Enzyme activity was reported as micrograms of tyrosine produced.

(d) Examination of Spoiled Vegetables and Fruits

Samples consisting of Onion, Tomato, Lemon, and Jaggery which were extensively damaged were collected and the spoiled parts were scrapped on a clean slide over drop of water. The amount was spread into a smear and stained with lacto phenol cotton blue and Gram stained.

(e) Production of Protease on Synthetic French Bean Meal Based Fermentation media

The axenic culture from the PDA plates were transferred to fresh PDA plates and allowed to form spores by incubating the plates at room temperature. The spores were suspended in 10mlbuffer mixed and poured into fermentation flask containing synthetic French bean meal fermentation medium. Flasks were grown at room temperature at static conditions for prolonged period of 360 hrs. The sample were intermittently collected in an aliquot of 10ml and centrifuged at 5000 rpm for 10 min. The supernatant was collected and considered as crude enzyme it will also suitably diluted and referred to as diluted enzyme. The amount of enzyme activity was determinant as further procedure described in methodology (Paranthanam, 2009).

(f) Production of protease by altered synthetic Fermentation media

Fermentation medium condition was altered by addition of sodium chloride as described in methodology. The Aspergillus isolate was grown on PDA as described earlier and 10 ml suspension of the spores were

inoculated into modified synthetic fermentation media. The flasks were incubated at room temperature for 72 hrs. The fermented matter was centrifuged, as described earlier, and the supernatant was used for enzyme estimation.

4.2. Production from Trichoderma sp.

The purpose of this study was to increase alkaline protease production by subjecting indigenous protease producing strain Trichoderma reesei MTCC-3929 to improvement by random UV mutagenesis and N-Methyl-N'-nitro-N-nitroso guanidine (NTG) treatment (Djamel et al., 2009). Mutants were screened as protease producers on the basis of zone of clearance on skimmed milk agar plates. UV-8 mutant showed 9 mm clear zone diameter and activities of 199.6 and 552.6 U/ml for submerged fermentation (Smf) and solid state fermentation (SSF), respectively. UV-8 further mutated by NTG to produced NTG-17 mutant with zone of clearance 13mm diameter. Compared to wild strain, NTG-17 mutant was found to produce 2.6 and 2.2-fold more activities in SmF and SSF, respectively. Thus these findings have more impact on enzyme economy for biotechnological applications of microbial proteases.

4.2.1. Materials and Methods

(a) Microorganism and growth media

The T. reesei MTCC-3929 which was procured from Microbial Type Culture Collection (MTCC), Chandigarh (India). It was grown on Potato Dextrose agar (PDA) at 30°C, and then stored at 4°C. PDA slants, incubated for 7 days, were used for the preparation of the inoculum.

(b) Preparation of spore suspension

Ten milliliters of 0.85% saline containing 0.1% Tween-80 was transferred to a sporulated (7 day old) PDA slant culture of T. reesei. The spores were dislodged using an inoculation needle, under aseptic conditions, and the suspension was used for its spore count by serial dilution and plating on PDA agar medium.

(c) Protease screening

Protease production by T. reesei (wild and mutants) were tested on skimmed milk agar plates containing (g/L) 4 potato infusion, 20 dextrose, 10 skimmed milk, 20 agar (pH 5.6).

(d) Enzyme assay

The protease activity was determined by caseinolytic assay method of (Kanekar et al., 2002). The cell free supernatant (1ml) was mixed with 4ml of casein (0.625% w/v) and incubated at 40°C for 30min. The reaction was stopped by addition of 5ml of 5% trichloro acetic acid. Enzymatically hydrolyzed casein was measured by modified Folin Ciocalteu method, against casein treated with inactive enzyme as blank. A standard graph was generated using standard tyrosine solutions of 5–50 µg ml-1. One unit of protease activity was defined as the amount of enzyme which liberated 1 µg tyrosine per min at 40°C.

4.2.2. Isolation and selection of mutants

(a) UV irradiation

Four ml of the spore suspension containing 107spores/ml was pipetted aseptically into sterile petri-dish of 80 mm diameter having a flat bottom. The exposure of spore suspension to UV light was carried at distance of 30 cm away from UV lamp (15W, 2537A0). The exposure times were 5, 10, 15, 20 and 25 min. Each UV exposed spore suspension was stored in dark overnight to avoid photo reactivation, then was serially diluted in saline and plated on PDA medium. The plates were incubated for 7 days at 30°C and the numbers of colonies in each plate were counted. Each colony was assumed to be formed from a single spore. Mutants were selected from the plates showing less than 1% survival rate and screened for protease production on skimmed milk agar plates (Djamel et al ., 2009).

(b) NTG treatment

The best UV mutant (UV-8) was used for NTG treatment. The spore suspension was prepared in the same manner as described earlier and calculated the spore count. To 9 ml of spore suspension (106 spores/ml), 1 ml NTG solution (10 mg/ml in sterile water) was added. The reaction was allowed to proceed at 30°C, 120 rpm. Samples were withdrawn from the reaction mixture at intervals of 20, 40, 60, 80 and 100 min and immediately centrifuged for 10 min at 5000 rpm and the supernatant solution was decanted. Cells were washed three times with sterile water and resuspended in 10 ml of sterile saline. The samples were serially diluted in the same saline and plated over PDA as mentioned earlier. NTG mutants were selected from the plates showing less than 1% survival rate and screened for protease production on skimmed milk agar plates. When compared to wild strain, NTG mutant showed 2.6 and 2.2-fold higher protease activities in Smf and SSF. Likewise, but higher fold (3.5) protease activities were reported by UV/NTG mutant of Bacillus pumilus (Wang et al., 2007).

(c) Protease production

Hyper producing mutant of UV, NTG treatment and a wild strains were inoculated separately in Smf and SSF medium. The liquid medium was prepared which containing (g/l) 5 wheat bran, 10 soybean meal, 0.1 yeast extract, 2 KH_2PO_4, 4 K_2HPO_4, 0.5 NaCl, 0.1 $MgSO_4$, 2 $CaCl_2$ (pH 7) and the solid medium contained 10 g wheat bran with 10 ml water. Both fermentations were performed at 30°C up to 5 days under shaking and static conditions for Smf and SSF, respectively. For solid medium, the fermented koji was mixed with 100 ml water and kept for 2hrs at room temperature. After 2hrs, it was filter off through cotton filter. The filtrate was used for protease activity measurements.

V. DOWNSTREAM PROCESSING

A wide range of techniques have been used for the recovery of the product from the fermented substrate depending on the source (liquid or solid form), scale of operation, enzyme stability etc. The objectives of a design for the purification have been based on the process parameters such as high degree of purity, high overall recovery of activity of enzyme, and reproducibility. Since NPRs are extremely sensitive towards auto digestion, conditions were chosen focusing on stabilization.

As an extracellular enzyme, for SSF, the fermented solids are crumbled and extraction in either a batch or continuous process. This is followed by enzyme leaching with water, aqueous buffers, diluted solutions of salts (e.g. 0.9% sodium chloride), glycerol (1%), or diluted (0.1%) solutions of non-ionic detergents such as Triton X-100 and Tween 20, etc. In liquid preparations, the enzyme is stabilized against chemical and microbial denaturation and degradation by the addition of high concentrations of salts such as ammonium sulphate and preservatives such as glycerol to increase product shelf life. The pH of the liquid should be adjusted.

There are activating effect of divalent cations (Mg^{2+}, Ca^{2+}, Fe^{2+}) and inhibiting effect of certain chelating agents (EGTA, EDTA) on metalloprotease (Sumantha et al., 2005). Inhibitory effect of Co^{2+} has also been reported. Specific NPRs might also be stabilized by the addition of divalent cations, which act as either oxidizing or reducing agents (Sumantha et al., 2006). While some of these additives may also act as preservatives, specific anti-microbial preservatives are also added. NPRs that show greater stability in solid form like thermolysin are spray-dried to obtain the powdered form, while others are used as liquid. In addition, the effects of solvent composition on the activity and reported remarkable activation and stabilization have been evaluated by high concentrations of neutral salts, crystallographic analysis in the presence of 4 M NaCl, and the activation by cobalt-substitution of the catalytic zinc ion, and inhibitory effects of alcohols (Kusano et al., 2006).

Purification of proteases to homogeneity is a prerequisite for studying their mechanism of action. Vast numbers of purification procedures for proteases, involving affinity chromatography, ion exchange chromatography, and gel filtration techniques, have been well documented. Cooperative enzymes system is promising, considering that purification of different enzymes is generally tedious and costly when they are purified individually from different fermentation systems, and their combined activities maybe greater than the sum of their individual activities. There're few reports on cooperative enzymes comprising alkaline or mixtures of alkaline and NPRs without stabilizers.

VI. APPLICATIONS

Proteases are one of the most important classes of proteases from an industrial point of view, occupying a major share of the total enzyme market. Use of proteases as active ingredients in detergents is the largest application of this enzyme. They are also widely used in leather industry, medical diagnostics, recovery of silver from X-ray films, silk degumming, and food and feed industry etc. Due to their vast applications in the industrial processes, many companies started manufacturing them at commercial level.

6.1. Detergent industry

Alkaline proteases have contributed greatly to the development and improvement of modern household and industrial detergents. They are effective at the moderate temperature and pH values that characterize modern laundering conditions in industrial & institutional cleaning. Various enzymes used in laundry industry are proteases, lipase, cellulases, amaylses etc (Ito et al., 1998). Of these, alkaline protease find a major application as detergent additives because of their ability to hydrolyze and remove proteinacious stains like blood, egg, gravy, milk etc in high pH conditions (Saeki et al., 2007). Proteases and other enzymes used in detergent formulations should have high activity and stability over a broad range of pH and temperature. The enzymes used should be effective at low levels (0.4–0.8%).

One of the most difficult design challenges that biotechnologists face is that the protease should be compatible with various commercially available detergents and its function is not hampered in the vicinity of typical detergent ingredients, such as surfactants, builders, bleaching agents, bleach activators, fillers,

fabric softeners and various other formulation aids. Recently, alkaline proteases from Bacillus cereus, Bacillus pumilus strain CBS, Streptomyces sp. strain AB1, Bacillus licheniformis, Aspergillus flavus, Aspergillus niger, Bacillus brevis, Bacillus subtilisAG-1 have exhibited excellent detergent compatibly in the presence of certain stabilizers such CaCl2 and glycine. To survive the extremes of high alkalinity and chelator concentration in detergents, subtilisins have been improved with respect to their thermo stability, resistance to chelators (Bryan, 2000). To prevent the loss of activity, several oxidatively stable serine proteases (OSPs) suitable for use in detergents have been isolated from alkaliphilic Bacillus strains.

6.2. Leather industry

Soaking, dehairing of hides and skins and bating have traditionally being carried out by using different chemicals which poses a high tannery waste pollution threat. Hence, proteases with a pH optimum around 9–10 are widely used in soaking to facilitate the water uptake of the hide or skin. Alkaline proteases with elastolytic and keratinolytic activity are used for dehairing and bating process to obtain a desired grain, softness and tightness of leather in a short time. Alkaline proteases with keratinolytic activity have been reported for remarkable dehairing properties. A novel protease showing keratinolytic activity from B. subtilis has been studied as apotential for replacing sodium sulfide in the dehairing process of leather industry (Arunachalam et al., 2009). Verma et al., 2011 showed the use of protease from Thermoactinomyces sp. RM4 for dehairing goathides.

6.3. Chemical Industry

A high stability in the presence of organic solvents is a feature which is highly desired in applications involving biocatalysis in non-aqueous medium for peptide synthesis. Alkaline proteases from Aspergillus flavus, Bacillus pseudofirmus SVB1, Pseudomonas aeruginosa PseA have shown promising results for potential of peptide synthesis due to their organic solvent stability (Sen, 2011). In addition to demonstrating high organic tolerance, alkaline proteases from B. pumilus strain CBS and Streptomyces sp. strain AB1 are potential strong candidates for use in peptide synthesis in low water systems. Apart from peptide synthesis, they have also been reported for organic synthesis. Alcalase, an industrial alkaline protease has been used for the synthesis of Bz-Arg-Gly-NH (N-benzoylargininylglycinamide), precursor dipeptide of RGDS (Arg-Gly-Asp-Ser) catalyzed in water/organic co-solvent systems (Yang et al., 2006). Synthesis of 2H-1-benzopyran-2-one derivatives using alkaline protease from Bacillus licheniformis has been reported by Wang et al., 2011. Regio selective synthesizes of polymerizable vinylguaifenesin ester has been studied by an alkaline protease from Bacillus subtilis.

6.4. Medical Uses

The use of immobilized alkaline protease from Bacillus subtilis possessing therapeutic properties has been studied for development of soft gel-based medicinal formulas, ointment compositions, gauze, non-woven tissues and new bandage materials (Davidenko, 1999).Oral administration of proteases from Aspergillus oryzae has been used as a diagnostic aid to correct certain lytic enzyme deficiency syndromes. Alkaline-fibrinolytic protease has been reported to preferentially degrade fibrin suggesting its future application in thrombolytic therapy and anticancer drugs (Mukherjee et al., 2011).

6.5. Waste management

Wastes from poultry processing industry and leather industry are recalcitrant to commonly known proteolytic enzymes due to presence of keratin-rich wastes whose polypeptide is densely packed and strongly stabilized by several hydrogen bonds and hydrophobic interactions in addition to several disulfide bonds. Chemical and mechanical hydrolysis of keratin wastes is successful but they have several disadvantages of being energy intensive, polluting and leading to loss of essential amino acids. Hence, enzymatic degradation using alkaline proteases with keratinolytic activity (keratinases) is an attractive method (Ramnani et al., 2005). Bacillus species is the most widely reported bacterial source of keratinases for feather degradation (Cail, 2011). Other reported bacterial sources of keratinases are Pseudomonas sp. MS21, Microbacterium sp., Chryseobacterium sp. and Streptomyces sp...Fungal keratinases from Aspergillus oryzae, Chrysosporium indicum, Trichophyton mentagrophytes, Microsporum sp. Trichophyton sp., Aspergillus terreus, Scopulariopsis sp. Fusarium oxysporum have also been studied towards the degradation of keratin.

6.6. Feed and Food industry

Alkaline proteases are widely used for production of protein hydrolysates for more than 40 years. Hydrolysates can be used as additives to food and mixed feed to improve their nutritional value. In medicine, they are administered to patients with digestive disorders and food allergies (Neklyudov et al., 2000). Protein hydrosylates can be obtained from a variety of substrates such as whey, meat, soy and casein. It is well known that meat hydrolysates usually taste bitter when the degree of hydrolysis (DH) is above the

10% required for satisfactory solubilization. With Novozymes's Flavourzyme, it is possible to degrade the bitter peptide groups and obtain a degree of hydrolysis of 20% without bitterness. Alkaline proteases also find their use in meat processing. SEB Tender 70, commercially available proteases is extensively used in meat tenderization to break down collagens in meat to make it more palatable for consumption.

6.7. Silver Recovery

Silver is one of the precious and noble metals used in large quantities for many purposes, particularly in the photographic industry. The waste X-ray/photographic films containing black metallic silver spread in gelatin are very good source for silver recovery compared to other types of film. The amount of silver in the X-ray film varies between 1.5 and 2.0% (w/w). Various traditional methods for silver recovery are burning the films directly, oxidation of the metallic silver following electrolysis, stripping the gelatin-silver layer using different chemical solutions. But this method posses serious environmental considerations. For this reason, enzymatic hydrolysis of gelatin is an alternative option to minimize the impact on environment. Successful recovery of silver from X-ray films have been reported by alkaline proteases derived from Bacillus subtilis, Conidio boluscoronatus, Streptomyces avermectinus. Recovery of silver and polyethylene terephthalate from used lith film for printing using the thermo stabilized mutant enzyme of the alkaline protease from alkaliphilic Bacillus sp. B21-2 has also been reported (Ishikawa, 2004).

VII. CONCLUSION

Proteases have found a wide range of applications in various industries such as food, pharmaceutical, cosmetic, etc. and have been widely commercialized by various companies throughout the world. Though the production of these enzymes has been improved significantly by the utilization of hyper-producing strains of fungi and bacteria and genetically modified microbes as well, efforts are still being done to find newer sources of enzymes, better production techniques and novel applications of these enzymes in unexplored fields.

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