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Natural Material Role in Production, Activation and Stabilization of Alkaline Protease Produced from a New Isolated Geobacillus caldoxylosilyticus IRDO

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Abstract: A new strain IRDO alkalophilic Geobacillus caldoxylosilyticus was isolated from a Tikehau pond in the Atoll, Polynesian France. It was identified on the basis of 16s rRNA gene homology, GC, where it was 95% related to this strain. This strain is unique in that it could grow easily and produced alkaline protease in presence of one gram of wheat bran or rice stalk without any supplements. Growth of Geobacillus caldoxylosilyticus under solid state fermentation (SSF) using wheat bran or rice stalk as the sole carbon and nitrogen sources produced appreciable levels of alkaline protease (1890 U/gm, 300 U/gm) respectively. The optimized parameters for enzyme production were studied. The neem extract played a significant role in activation and stability of the partially purified alkaline protease. The hydrolytic activity of the alkaline protease towards different natural substrates improved in presence of 0.05 % neem extract. This enzyme showed keratinaceous hydrolytic ability towards human hair and nails. The ability of the enzyme to remove blood stains increased significantly in the presence of neem extract.

Key Words: Geobacillus caldoxylosilyticus, Alkaline protease, Neem extract

INTRODUCTION

Proteases are the one of the most industrial enzymes accounting for nearly 60% of the total world wide enzyme sales.\[1,2] Although protease production is an inherent property of all organisms, only alkaline proteases from high yielding strains have been studied extensively. In view of its possible applications, alkaline protease from extremophilic microorganisms could produce commercially in high yield at a low cost method. Recently, production of extracellular alkaline protease has been optimized from alkalophilics bacilli.\[3,4] Alkalophilic microorganisms are considered as extremophiles because they have pH optima for growth in an alkaline range 9.0-10.0.\[5]

Cheaper of both carbon and nitrogen sources are the attraction key for commercialization of the production process and thus, ability of the microbial agent to grow and produce enzymes, using these sources has been arguably a point of interest.\[5,6] In literature, there are few instances of the usefulness of the cheap carbon and nitrogen for the alkaline protease production. For example, an extracellular alkaline protease from Bacillus horikoshii, was optimally produced when grown in soybean meal. Maximum enzyme activity was observed at pH 10.5 which is essential characteristic for the use of alkaline protease as laundry.\[7]

The present work has focused on the production of extracellular alkaline protease from alkalophilic bacteria named IRDO. It was related to a new strain Geobacillus caldoxylosilyticus. This strain has the ability to grow and produced substantial amounts of alkaline protease under solid state fermentation using wheat bran (WB) as the sole carbon and nitrogen source moisture with the distilled water. The production was improved by the addition of plain flour and curcuma. Many authors reported in the curcuma as plant extract possessed proteolytic activity\[8] but no one talk about it in alkaline protease production. The partially purified enzyme showed a great increase of activity and stability in the presence of neem extract.

This enzyme showed great hydrolytic ability towards crude materials. It also removed the blood stain especially in the presence of neem extract.

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MATERIALS AND METHODS

Isolation Method: Isolate IRDO was isolated from a pond named MARAKOPARA in the Atoll Tikehau (French Polynesia). The samples were stored in seawater at 4 °C until processing.

The basal medium (BM) contained (l distilled water): 0.2 g NH₄Cl, 0.13 g KH₂PO₄, 0.15 g Na₂HPO₄.₃H₂O, 9.5 g NaCl, 0.8 g Na₂SO₄, 3.2 g sodium lactate, 10 ml trace mineral element solution[9]. The pH was adjusted to 7.0 with 10 M KOH, five-milliliter aliquots of medium were dispensed into Hungate tubes and 40 ml aliquots were dispensed into serum bottles (100 ml) under a stream of N₂-CO₂ (80:20, v/v), and the sealed vessels were then autoclaved for 45 min at 110 °C. Prior to inoculation, tree sterile stock solutions NaHCO₃ (10% w/v), CaCl₂.2H₂O (0.3 % w/v), and MgCl₂.2H₂O, MgSO₄.7H₂O (2% and 0.7 % w/v) were injected to respective final concentrations of 0.3 %, 0.01 %, 0.08 %, 0.03 % (w/v). Before inoculation with 2 ml of sample, the gas phase of tubes and serum bottles was purged with a gas stream of N₂-O₂ (99:1, v/v). The serum bottles containing BM were incubated at 45 °C to initiate an enrichment culture. The culture was purified by using repeated Hungate roll tube method with BM solidified with 15 g /l agar.

Production of Alkaline Protease in Different Substrate: One gram of these substances, wheat bran, rice stalk, starch, rice, plain flour or maize flour in 50 ml distilled H₂O (pH 7.0) in 250 ml Erlenmeyer flask. The flasks were autoclaved at 121 °C for 30 min. The preparation of inoculum was done by scratched 24h old slants with 2 ml sterilized distilled water, and transferred to 250 ml Erlenmeyer flask, mixed thoroughly and incubated in a rotary shaker at 150 rpm for 24 h at 30 °C. The growing bacteria were subculture one time into a new medium and the alkaline protease production was determined.

Solid State Fermentation: These studies on Geobacillus caldioxyslyticus were performed using commercially available wheat bran or rice stalk as the solid substance. Ten grams of particle size< 420 µm taken in 250 Erlenmeyer flasks were mixed with 8 ml distilled water thoroughly and incubated at 121 °C for 30 min. They cooled to room temperature and each flask was inoculated with 2 ml inoculums.

Extraction and Enzyme Recovery: Protease from the WB was extracted by the simple method of extraction using distilled water as extractant[10]. Ten volumes of distilled water per gram WB (based on initial dry weight of the substrate) was added to the fermented media and the extraction was performed by agitation at room temperature in a rotary shaker for 60 min at 150 rpm. The slurry was then squeezed through wound sheet and clarified by centrifugation at 5,000 rpm at 4 °C for 15 min. The clear supernatant was used as crude enzyme for protease assay.

Assay of Enzyme Activity: Caseinase activity was estimated according to the method of, [11] by determining the rate of hydrolysis of 1 ml of 1.5% (w/v) casein solution in glycine NaOH buffer 0.05M at pH 8.5 with 1.0 ml diluted enzyme after incubation for 30 min at 40 °C.

One unit (U) of enzyme activity was taken as the amount of enzyme that liberated 1µg of tyrosine per ml per minute.

Enzyme activity was expressed as units per gram initial dry substrate (U/g WB) in the solid state experiments and (U/ml) in the others.

Optimization of Process Parameters for Protease Production: The parameters studied included initial moisture content (50-150%), incubation time (0-72 h), incubation temperature (35-50 °C), additional of plain flour at (0.05- 0.25 g/ g WB), curcuma (0.005-0.02g / g WB).

16S rRNA Sequence: The methods for the purification and extraction of DNA and the amplification and sequencing of the 16S rRNA gene have been described previously[12], except for the use of the primer Rd1 (5'-AAGGAGGTGATCCAGCC-3') instead of R6. The samples were loaded onto an Applied Biosystems 373XL sequencer and run for 12 h on a 4.5 % denaturing acrylamide gel by Genome Express Co (Grenoble, France). Sequence data were imported into the sequence editor BioEdit v5.0.9,[13] the base calling was examined and a contiguous consensus sequence was obtained for each isolate. The full sequence was aligned using the Ribosomal Database Project’s (RDP) Sequence Aligner Program[14]. The consensus sequence was then manually adjusted to conform to the 16S rRNA secondary structure model[15]. A non-redundant blastn search[16] of the full sequence through GenBank[17] identified its closest relative. Sequences used in the phylogenetic analysis were obtained from the RDP[14].
and GenBank\textsuperscript{[17]}, Positions of sequence and alignment ambiguity were omitted and the pair-wise evolutionary distances based on 1210 unambiguous nucleotides were calculated using the method of \textsuperscript{[18]}. Dendrograms were constructed using the neighbour-joining method\textsuperscript{[19]}. Confidence in the tree topology was determined by using 100 bootstrapped trees.\textsuperscript{[20]}

**DNA-DNA Hybridization**: DNA-DNA hybridization was carried out by the Identification Service of the DSMZ (Braunschweig, Germany).

**Thermal Stability**: Profile was studied by incubating the enzyme preparation at various temperatures, (45, 50, and 55\(^\circ\)C) in glycine NaOH buffer 0.05M, pH 10 for different time period (15-60min) and the residual activity was measured at 60\(^\circ\)C.

**Preparation and Extraction of Aqueous Neem Extract**: Fresh neem fruits were collected from Dubai (United Arab Emirates) and were manually separated into seeds and seed coats and milled. According to the method \textsuperscript{[21]} with some modifications 5 g of defatting ground seeds were extracted with 200 ml of distilled water at 85\(^\circ\)C for 3 h. After filtration, the extract was dialyzed against distilled water for 48 h, dried and weighed. Total carbohydrate and soluble protein of the extracted seeds were determined by the method of \textsuperscript{[22,23]}

**Hydrolysis of Protein Substrates**: Enzyme activity with various protein substrates including casein, bovine albumin, gelatin, collagen and keratin was assayed by mixing 0.125 mg of the enzyme and 2ml of assay buffer containing (2.5 mg substrates /ml). After incubation at 50 \(^\circ\)C for 30 minutes, the enzyme activity was estimated according to the method of \textsuperscript{[24]}. Same experiment was repeated with the addition of 0.025% neem extract.

**Application of the Enzyme in Removing Blood Stains**: Human blood (100 \(\mu\)l) was applied on 100% cotton fabrics, air dried followed by oven drying at 95-100 \(^\circ\)C for 10 min. The enzyme only 300 \(\mu\)l), seed neem extract only (300 \(\mu\)l), the enzyme mixed with seed neem extract (300 \(\mu\)l equivalent to 20 U/ml), the enzyme mixed with neem extract in 1 \% Ariel detergent (300 \(\mu\)l equivalent to 20 U/ml), the enzyme in 1\% detergent (300 \(\mu\)l equivalent to 20 U/ml) were applied to the spots, and after 5 min it was washed with water. During the washing, scrubbing and use of detergent was avoided and the process was conducted at room temperature (24 \(^\circ\)C).

**Degradation Ability Against Hair and Nails**: The nails or hair (0.01 gm) incubated (at 40 \(^\circ\)C for 24 h) with one ml distilled water, one ml enzyme (100 U/ml), one ml neem extract only (0.05\%), and one ml enzyme (100 U/ml) +neem extract.

### RESULT AND DISCUSSIONS

A new isolated *Geobacillus caldoxylosilyticus* was identified on the base of the nucleotide sequence of 16S rRNA gene. The analysis of most recent 16S rRNA gene sequences available from the RDP and GenBank revealed that strain IRDO belonged to the genus *Geobacillus*, *Geobacillus caldoxylosilyticus* being its closest phylogenetic relative (similarity of 95\%). The level of DNA-DNA relatedness between isolate IRDO and *Geobacillus caldoxylosilyticus* was 26.8\% and therefore revealed that isolate IRDO should be assigned to a novel species of the genus *Geobacillus*\textsuperscript{[24]}. This study was initiated with the aim of isolating a new indigenous alkalophilic bacterial strain that are adapted to grow and produce alkaline protease even in the absence of any supplements. The strain could grow easily in presence of one gram of these substances: wheat bran, rice stalk, starch, rice, plain flour or maize flour in 50 ml distilled water using shaken culture technique at 150 rpm. The alkaline protease test showed that the medium contained the wheat bran produced (1.4 U/ml) of this enzyme, followed by rice stalk (0.35 U/ml) and plain flour (0.2U/ml). The remainder substances showed marginal amount of activity.

According to the previous results, different concentrations wheat bran or rice stalk (5-20 gm) in 10 ml distilled H\(_2\)O were used under SSF. The maximum activities (1890 U/gm, 300 U/gm respectively) were obtained at 10 gm. The following experiment was achieved in the presence of 10 g WB as the sole carbon and nitrogen sources. The results showed that the optimum production of the alkaline protease (3706 U/g) reached at 45 \(^\circ\)C and pH 9 after 48 h.

The ability of the strain to grow and produce substantial level of alkaline protease using WB as a substrate even in the absence of any supplements makes it very interesting. \textsuperscript{[25]} showed that *Bacillus pseufodformus* AL-89 produced high level of protease activity in feather medium. It was reported that maximum yields of the alkaline protease produced from a newly isolated *Bacillus sp.* (429.041 and 168,640 U/g) were achieved by employing wheat bran and lentile husk as substrates.\textsuperscript{[26]} The WB media was optimized and showed its maximum activity at 45 \(^\circ\)C and pH 9 after 48h. This result confirmed that there is no relationship between the growth and the enzyme
Fig. 1: Effect of plain flour concentration on alkaline protease production from Geobacillus caldoxylosilyticus IRDO grown under SSF condition.

Fig. 2: Effect of different concentrations of curcuma on alkaline protease production from Geobacillus caldoxylosilyticus IRDO grown under SSF condition.

Fig. 3: Effect of temperature profiles on the activity of alkaline protease (■) in the presence and absence of 0.05% neem extract (•).

Fig. 4: Effect of addition of (0.05%) from Henna, Curcuma, Ginger, Neem seeds and Cumin in alkaline protease activity and protein content, the control referred to the alkaline protease only.

Fig. 5: Alkaline protease activity against different natural substrates.

Production. The optimum production of novel alkaline protease from marine Engyodontium album BTMFS10 under SSF using wheat bran was at 25 °C, pH 11 for 72 h.\(^{[27]}\). The study of different moisture content showed that the maximum activity (4600 U/g) was at 150%. This result is agreeable with earlier studies,\(^{[28]}\) where it was reported that optimum initial moisture for Rhizopus oryzae alkaline protease production was 140%. Addition of different concentrations of plain flour (Fig 1.) showed that the maximum activity (8012 U/g) at 0.2 g plain flour/g WB. The level of protease product by Geobacillus caldoxylosilyticus IRDO greatly increased by the
Table 1: Thermal stability of the enzyme with neem extract at 0.05 % (E+N) and native alkaline protease (E) Relative values of alkaline protease activity expressed in (%) obtained by Beridge's method.

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Fig. 6: Test for degradation ability of Geobacillus caldoxylosilyticus alkaline protease against human nails and hair. 1. water + nails. 2. alkaline protease + nails. 3. neem extract + nails. 4. alkaline protease + neem extract + nails. 5. water + hair. 6. alkaline protease + hair. 7. neem extract + hair. 8. alkaline protease + neem extract + hair.

Fig. 7: Washing performance of alkaline protease from Geobacillus caldoxylosilyticus. A. Cloth stained with blood, B. blood stained cloth washed with enzyme only, C. blood stained cloth washed with neem extract, D. blood stained cloth washed with detergent, E. blood stained cloth washed with enzyme and detergent only. F. blood stained cloth washed with enzyme, detergent and neem extract.

addition of plain flour, as it is reach in carbohydrate and protein. It was reported that the production of alkaline protease on wheat bran supplemented with soy protein. The supplementation of the media with different concentrations of curcuma recorded its maximum activity (13825 U/g) at 0.015 g/g WB (Fig 2.). Many authors reported in the curcuma as plant extract possessed proteolytic activity but no one talk about it in alkaline protease production.

The partially purified alkaline protease was obtained at 40-60 % ammonium sulfate precipitation and achieved 15-fold purification. The enzyme is active over a broad range of pH (7.0-11.0) displaying over 84% of its maximum activity between pH (7.0-11.0). The optimum enzyme activity was observed at pH 9.0-10.0 (data not shown).

The temperature profile (Fig 3.) of Geobacillus caldoxylosilyticus showed its maximum protease activity at 55-60 °C however at 70 °C and 80 °C the enzyme retained 82.07 % and 72.75 % of its activity. According to the following experiments 0.05 % neem extract was mixed with the enzyme, the result (Fig 3.) showed that the maximum enzyme activity was converted to 70 °C. This result showed that the neem extract played a vital role in maintaining the active conformation of the enzyme at high temperatures. The result could be attributed to the presence of Ca\(^{2+}\) and carbohydrate as major components in neem extract as it will be discussed later. The enzyme was active at wide range of pH and temperature in the absence of Ca\(^{2+}\) ion. These properties make protease an ideal candidate for detergent application. The optimum assay parameters for alkaline protease activity of Penicillium sp. were: pH 9.0 and 45 °C. The test for enzyme activation by adding 0.05 % extract of Henna, Ginger, neem seeds, curcuma, and cumin, was recorded in (Fig 4.).The results showed that all the tested substances led to increase in enzyme activity especially in case of neem extract, where it was increased the enzyme activity to 170 % (The alkaline protease test was negative in all the previous
substances). This property recommended the enzyme to be used safely in an industrial food application. The compositions of aqueous extract of neem extract were found to be: 67.2 \% protein, 21.2 \% carbohydrate and 4.5 mg/g calcium ions. Complete acid hydrolysis of aqueous extract afforded 19 \%  uronic acid, 48.3 \% galactose, 22.5 \% glucose, 27.3 \% arabinose, traces xylose and 0.8 \% rhamnose. The addition of the carbohydrate, protein, CaCl$_2$ (equivalent to 0.05 \% neem extract) individually to the enzyme showed that the carbohydrate led to 20 \% increase in enzyme activity, in contrast the protein showed 80 \% inhibition, while the CaCl$_2$ was effective in improving the activity to 126 \%.

The effect of alkaline protease activity in the presence and absence of neem extract on different natural substrates was studied. The results in Fig 5. showed that all the tested substrates incubated with neem extract and enzyme showed higher hydrolytic activity than the enzyme only. Also the protease reported a high level of hydrolytic activity against casein and poor to moderate hydrolysis of gelatin and bovine albumin. The results (Fig 6) showed that the neem extract played a significant role in improving the hydrolytic activity of the enzyme, furthermore the fact that this enzyme act on hair and nails suggest their ability to bind the keratinaceous solid substrates, this is a very important property for detergent application, because detergent enzymes are expected to act on proteinacious substrates attached to solid surfaces.$^{[31]}$

The thermal stability was the same in the presence and absence of Ca$^{2+}$, while it was improved clearly in the presence of neem extract in Table 1. Further understanding of the structure of this enzyme and the mechanism of its Ca$^{2+}$ independent thermal stability may give a better insight for the rational design of new alkaline proteases through protein engineering. Many author talk about stabilization of alkaline protease by the addition of Mn$^{2+}$ Ca$^{2+}$$^{[32]}$ but no one reported in the stabilization by the addition of neem extract. All the used laundry commercial detergent 1 \% led to enhancement in the enzymatic activity however the enzyme activity increase to 250, 150 and 130 \% in case of Tide, Extra and Ariel respectively. Similar results were reported by $^{[32]}$ where 1 \% of Tide and Ariel showed 125 \% and 111 \% increase in alkaline protease activity. Finally the enzyme showed cleaning powerful against blood stain, especially in the presence of neem extract (Fig 7).

**Conclusion:** The authors in this research try to produce substantial amounts of alkaline protease, they avoided the used of chemicals substances and preferred using waste and crude materials, as it is cheaper and available, The *Geobacillus caldoxylosilyticus* protease possesses maximum activity at high pH and temperature range that indicates its usefulness in wide range of temperature wash programs. Also the results showed that the neem played a significant role in improvement the alkaline protease properties. This recommended the enzyme in industrial food application. The enzyme ability to bind the keratinaceous solid substrates makes it extremely attractive for detergent. All the previous work recommended our enzyme for industrial application.

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