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Isolation, screening and optimization of factors effecting protease production from *Comomonas kerstersii* KSM7

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Abstract: A protease producing bacteria was isolated from the soil and screened on skim milk agar medium for proteolytic activity. The highest proteolytic activity was exhibited by *Comomonas kerstersii* and was selected for further studies. The culture conditions for the bacteria were optimized by using different incubation time, temperature, pH, carbon source and nitrogen source. The result obtained in the present study revealed that higher production of protease can be optimized at 72 hrs of incubation period at 60^0C temperature and pH 10 by utilizing carbon source as glucose and nitrogen source as meat extract. Partially purified protease with molecular weight of ~25 kDa was observed in SDS-PAGE.

Key Words: Alkaline protease, 16s r RNA, optimum culture conditions, pH, temperature.

INTRODUCTION:

Currently, enzymes have attracted the world attention due to their wide range of industrial applications in many fields, including organic synthesis, clinical analysis, pharmaceuticals, detergents, food production and fermentation. Proteases are the most important class of industrial enzymes occupying a major share of 60% of the total enzyme market [1]. These biocatalysts hydrolyze peptide bonds in proteins and hence are classified as hydrolases and categorized in the subclass peptide hydrolases or peptidases [2]. Protease performs numerous varieties of activities in detergent, food, pharmaceutical, leather, laundry, food processing etc. These enzymes are widely used in dairy industry as milk clotting agent and meat tenderizing agent in food industry, reduction of tissue inflammation (clinical and medical) application [3]. Proteases are obtained from plant, animal and microbial sources. Microorganisms are the preferred source for obtaining proteases because of their fast growth

rate, easy to manipulate for getting highly stable enzymes through genetic engineering and requires shorter time for production and purification steps [4, 5].

Due to increased industrial demand for proteases it is expected that hyperactive strains will emerge and that the enzymes produced by new exotic microbial strains could be used as biocatalysts in the presently growing biotechnological era [2, 6]. Selection of the right organism plays a key role in high yield of desirable enzymes. For production of enzymes for industrial use, isolation and characterization of new promising strains using cheap carbon and nitrogen source is a continuous process. Available literature information indicates that, among all protease-producing microbial organisms, the *Bacillus* genus assumes importance because of its potential for production in large amounts [7, 8]. Moreover several medium components such as nitrogen and carbon sources, physiological factors such as pH, incubation temperature and incubation time, and biological factors such as the genetic nature of the organism influences the metabolic/biochemical behavior of the microbial strain and subsequent metabolite production pattern [2, 6, 9]. In general, no defined medium has been established for the best production of any metabolite because the genetic diversity present in different microbial sources causes each organism or strain to have its own special conditions for maximum product production [2]. Therefore, it is essential to have a detailed investigation on newly isolated microbial strain for production pattern under different environmental conditions and in an optimized pattern to achieve maximum production benefit [10, 11, 12].

The increasing importance of these enzymes and their numerous applications in different industries made us to investigate protease production from a new source. The isolation and characterization of new promising strains are possible ways to increase the yield of such enzymes. The aim of the present study was to isolate the bacteria from the soil, identification of the culture, screen the protease producing culture and optimization of cultural conditions for the production of protease.

MATERIALS AND METHODOLOGY

Collection of sample

Soil sample was collected from soil dump near field area of Padmashree Institute of Management and Sciences, Bangalore. 5gms of soil sample was taken and performed serial dilution.

Media

Nutrient agar media was prepared according to manufacturer's instruction and sterilized in autoclave at 121^0C for 15min.

Isolation of microorganism

The soil sample which is serial diluted was poured on to nutrient agar plates with respective dilutions from 10^{-2} – 10^{-7}. This was incubated for 24-48hours at 37^0C. Individual colonies identified were sub-cultured on to nutrient agar slants after 3 days to preserve pure cultures.

Screening and identification of bacterial isolate

The purified bacterial isolates were spread on plates composed of skimmed milk agar [0.5% (w/v) peptone, 0.5% (w/v) yeast extract, 1% (w/v) KH_2PO_4, 0.02% (w/v) $MgSO_4$, 1% (w/v) skimmed milk, 1% (w/v) NaCl and 1.5% (w/v) agar]. Isolates displaying relatively high proteolytic activity on these plates were further cultured in flasks for quantitative analysis. Using this process, one strain exhibiting the highest proteolytic activity was ultimately selected. The isolated strain was identified based on cellular morphology, gram staining, motility and biochemical profile tests [13]. The organism was further identified using 16S rDNA methodology.

DNA extraction and PCR Amplification:

The genomic DNA was isolated from the given organism using genomic DNA extraction Kit (Bhat Biotech). Amplification of the 16s rRNA gene was performed using the universal primers. The amplification was carried out in a Master cycler® Thermocycler (Eppendorf, Germany). PCR product of ~1500bp was purified to remove unincorporated dNTPS and Primers before sequencing.

Sequencing:

Both strands of the rDNA region amplified by PCR were sequenced by automated DNA sequencer -3037*xl* DNA analyzer from Applied Biosystems using BigDye® Terminator v3.1 cycle sequencing Kit (Applied Biosystems). Sequence data were aligned and dendrograms were generated using Sequence analysis software version 5.2 from Applied Biosystems. The sequences obtained for plus and minus strands were aligned using DNA baser software before performing the bioinformatics analysis.

Bioinformatics analysis:

Sequences were compared to the non-redundant NCBI database by using BLASTN, with the default settings used to find the most similar sequence and were sorted by the E score. A representative sequence of 10 most similar neighbours was aligned using CLUSTAL W2 for multiple alignments with the default settings. The multiple-alignment file was then used to create phylogram using MEGA5 software.

Production of proteases

The culture medium (90ml sterile broth) containing glucose (1.0g/l), peptone (10.0g/l), yeast extracts (0.2g/l), $CaCl_2$ (0.1g/l), K_2HPO_4 (0.5g/l) and $MgSO_4$ (0.1g/l) was inoculated with 10ml inoculum and incubated at 37^0C for 48 h in a shaking incubator (150rpm). After 48 h of incubation, the cells were harvested at 15000 rpm for 10 min and the clear crude supernatant was stored at 4^0C for further studies.

Protease assay

The enzyme was assayed in the reaction mixture containing 2.0ml of 0.5% casein solution in 0.1 M CO_3-HCO_3 buffer (9.0) and 1ml enzyme solution in a total volume of 3.0ml. The reaction were carried at 37^0C for 10 min and then terminated by adding 3.0 ml of 10% TCA and centrifuged at 10000 rpm for 15 min. One unit of enzyme (µg/ml/min) was defined as the amount of enzyme that liberated 1g tyrosine per ml per minute under assay conditions.

Protein assay

Total protein of the cell free filtrate was determined by the Lowry's method [14]. Bovine serum albumin (250µg/ml) was used as a standard.

Effect of incubation period, temperature and pH on protease production

The effect of incubation period for protease production was determined by incubating production medium at different incubation period viz. 12, 24, 48, 72 and 96h. Optimum temperature for protease production was achieved by incubating the culture medium at 25- 65^0C by the increment of 10^0C for 48h. The pH of the medium was adjusted before autoclaving from 5.0- 11.0 by the increment of 1.0 at 37^0C for 48h.

Effect of different carbon and nitrogen sources on protease production

The culture medium was added with different carbon source (2%) such as glucose, sucrose, maltose, lactose, mannitol and sorbitol for protease production. To study the effect of different nitrogen sources on protease production, nitrogen source (0.1%) such as ammonium chloride, ammonium sulphate, potassium nitrate, peptone, tryptone and yeast extract were used in culture medium.

Partial purification of protease enzyme

Partial purification of protease enzyme was achieved by ammonium sulphate precipitation followed by dialysis. 100 ml of cell free extract was saturated with ammonium sulphate up to 80%. The content was incubated over night and centrifuged at 5000 rpm for 20 min. Supernatant was collected and saturated up to 90% with ammonium sulphate. Then the content was centrifuged at 5000 rpm for 20 min and pellet was collected for further analysis. The enzyme mixture was transferred in a dialysis bag and immersed in phosphate buffer at 4°C for 24 hr. Buffer was continuously stirred using a magnetic stirrer throughout the process. Buffer was changed three times during the process in order to obtain proper purification.

Molecular weight determination

SDS-PAGE was carried out in 12.5 % resolving gel and 4.5 % stacking gel for determination of molecular mass as per the method of Laemmli., 1970 [15]. Protein bands were detected by destaining the gel in a methanol-acetic acid-water solution (4:1:5) after a staining process with 0.2% Coomassie brilliant Blue. The molecular mass of the partially purified protease was estimated using standard protein marker of known molecular weight (Bangalore Genei Pvt. Ltd., Bangalore, India).

Statistical analysis

All the experiments were carried out independently in triplicates and the data represented here are the mean value ± SD calculated using the Excel Spreadsheets available in the Microsoft Excel.

RESULTS AND DISCUSSION

Isolation and screening of bacterium for protease production

In the present study 7 isolates were obtained from soil sample and was screened for protease production by inoculating them in casein agar and skim milk agar. Among which 2 organisms (Sample A and B) showed zone of substrate hydrolysis. Later the bacterium showing highest zone of substrate hydrolysis (0.9mm) was named as KSM7 (Sample B) and selected for further studies.

Identification of bacterium

From the result, the bacterial isolate which showed highest starch hydrolysis was observed as gram positive, spore forming, rod-shaped, aerobic, catalase positive and oxidase positive. Molecular phylogenetic studies showed that the strain was a member of *Comomonas* species. A comparison of the DNA sequence with sequences in the NCBI database with BLAST software showed 98% sequence identity with the published 16S rRNA gene sequence of *Comomonas kerstersii* (Fig. 1, 2). The 16S rDNA sequence of the isolate was deposited at Genbank. This is probably a first report on the production of protease by using *Comomonas kerstersii*. Hence it is found to be having prestigious place in enzyme production technology as it serves as new source for various enzymes.

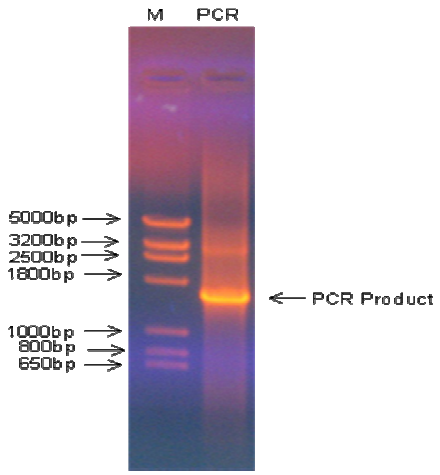

Fig. 1: PCR amplification of 16srRNA gene- 0.8% Agarose gel electrophoresis showed PCR product of 1.5 kb. M- DNA molecular size marker.

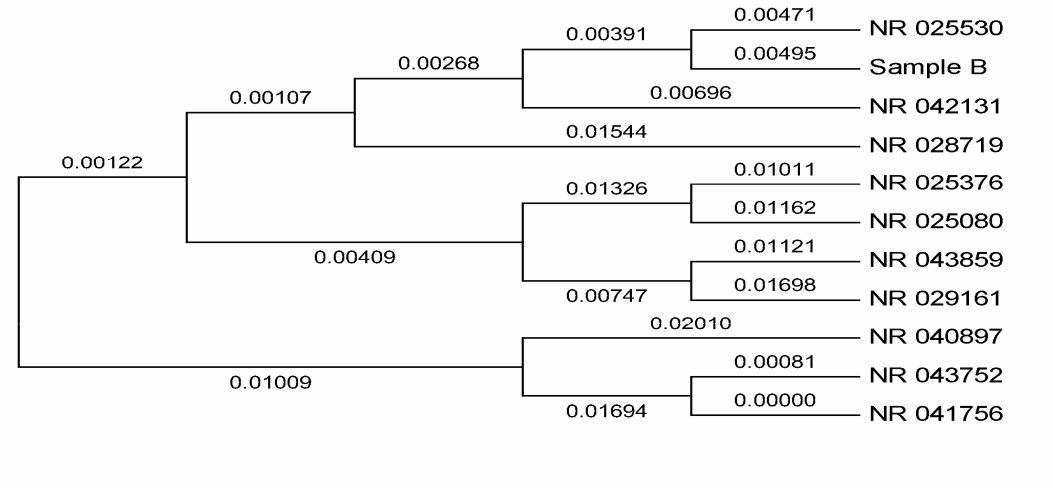

Fig. 2: Phylogenetic tree-

NR 028719- *Comamonas terrigena* strain IMI 359870
NR 025530- *Comamonas kerstersii* strain LMG 3475
NR 043859- *Comamonas odontotermitis* strain Dant 3-8
NR 025376- *Comamonas nitrativorans* strain 23310
NR 042131- *Comamonas aquatica* strain : LMG 2370
NR 029161- *Comamonas testosteroni* strain KS 0043
NR 025080- *Comamonas denitrificans* strain 123
NR 043752- *Acidovorax avenae* strain FC-143
NR 040897- *Giesbergeria giesbergeri* strain IAM 14949
NR 041756- *Acidovorax cattleyae* strain ICMP 2826

Optimization of cultural conditions

Culture conditions such as pH, temperature, source of carbon, nitrogen, protein and metal ions are known to influence the synthesis and secretion of extracellular enzymes by microorganisms [10, 11, 12]. Optimization of the culture conditions is hence, necessary in the selection of the bacterial source for industrial exploitation of their extracellular enzymes.

Effect of incubation period on protease production

Incubation period plays an important role in the maximum production of enzymes. *Comomonas kerstersii* KSM7 showed a gradual increase in protease titer from day 1 at pH 7.0 and 37 °C with the maximum at day 3 (2.0 µg/ml/min) (Fig 3). The protease production decreased after 96 hrs of incubation which may have been due to insufficient availability of some nutrients in the growth medium. Also the subsequent decrease in the enzyme units could probably due to inactivation of enzyme by other constituent proteases [16]. These results are in accordance with observations made by Qadar et al., in Bacillus sp. PCSIR EA-3 [17] and Srividya and Mala in *Bacillus sp.*Y [18] isolated from soil. A broad incubation period ranging from 48 to 70 h for maximum protease enzyme yield by *Bacillus* strains has been previously reported [19, 20].

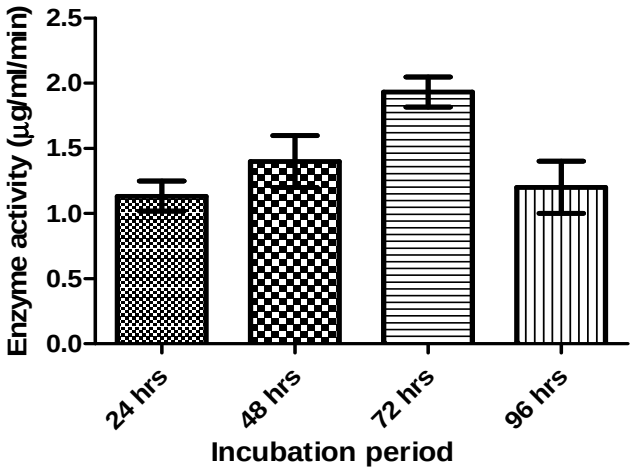

Fig. 3: Effect of incubation period on the activity of alkaline protease from *Comomonas kerstersii* KSM7

Effect of growth temperature on protease production

Temperature is one of the most critical parameters to be controlled in any bioprocess engineering. Optimum temperature for protease production varied from 34 to 60°C depending on the bacterial strain used [21]. In the present study, the optimum temperature for protease enzyme activity was found to be at 60^0C (Fig 4). Similar results were described for other *Bacillus* proteases. For example, the optimum temperature for protease from thermophilic *Bacillus* HS08 was 65ºC [22], and from *Bacillus* subtilis PE-11 and *Bacillus* majovensis was 60ºC [23, 24].

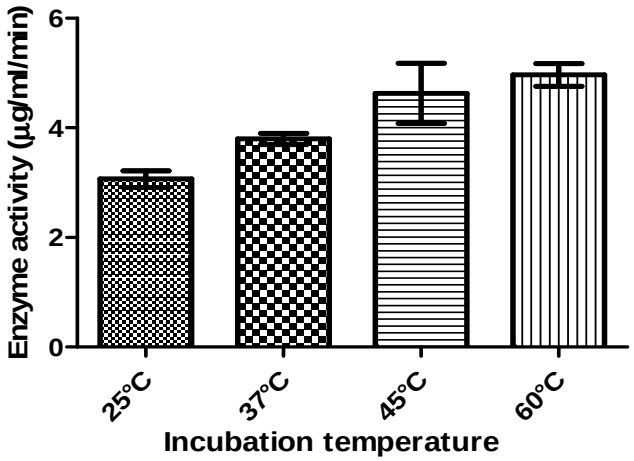

Fig. 4: Effect of incubation period on the activity of alkaline protease from *Comomonas kerstersii* KSM7

Effect of media pH on protease production

From a survey of literature it can be seen that the optimum pH range of alkaline proteases is generally between pH 9 to 11. In the present study, the optimum pH for protease activity was 9 although the enzyme was active in the pH range of 7- 12 (Fig 5). Alkaline proteases of *Bacillus subtilis* PE-11 and *Bacillus mojavensis* with similar properties have been reported by Adinarayana et al., and Qasim and Rani [23, 24].

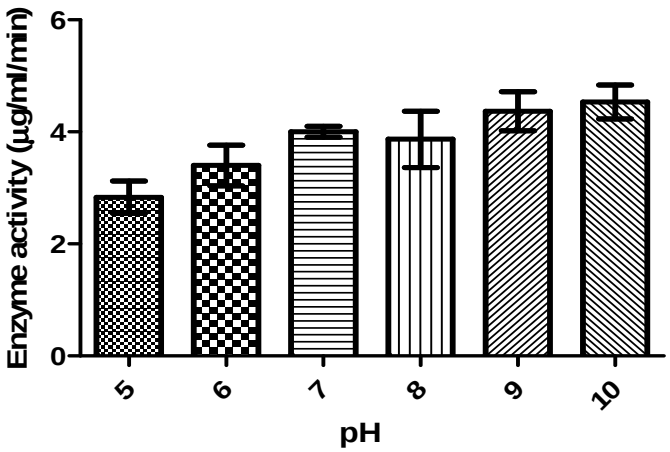

Fig. 5: Effect of incubation period on the activity of alkaline protease from *Comomonas kerstersii* KSM7

Effect of different carbon sources on protease production

Various carbon sources such as fructose, sucrose, galactose, lactose, and maltose were used to replace glucose which was the original carbon sourse in the medium. From the result it was found that, glucose and galactose was the best substrate for protease production when compared to other carbon sources. It showed maximum enzyme activity of 3.8 µg/ml/min (Fig 6). This was followed by lactose, maltose and sucrose. Similarly glucose was found to be the best carbon source for protease production by *Bacillus subtilis* [25] while starch was found to be best carbon source for protease production by *Bacillus licheniformis* S-40 [26].

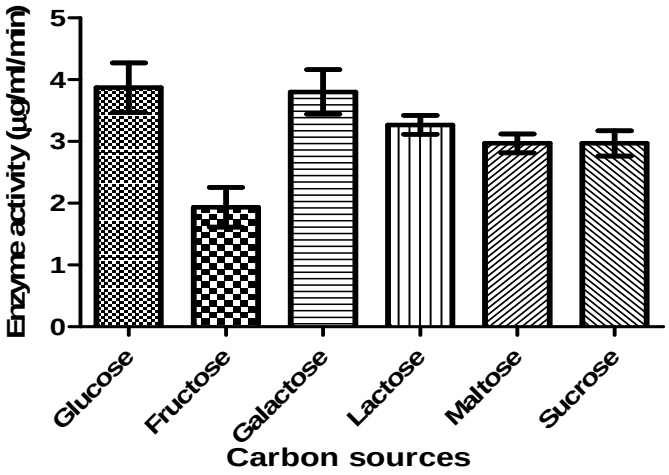

Fig. 6: Effect of incubation period on the activity of alkaline protease from *Comomonas kerstersii* KSM7

Effect of different nitrogen sources on protease production

Production of extracellular proteases has been shown to be sensitive to repression by different carbon and nitrogen sources [27, 28]. Among the tested nitrogen sources, supplementation of meet extract followed by peptone lead to optimum protease production by *C. kerstersii* KSM7 (Fig 7). These results are in agreement with the findings of Wang and Hsu [28]. It was observed that, medium supplemented with organic nitrogen sources supported higher protease production when compared to inorganic nitrogen sources. Similar reports were made by Narayana *et al*. and Narasimha *et al.* [29, 30].

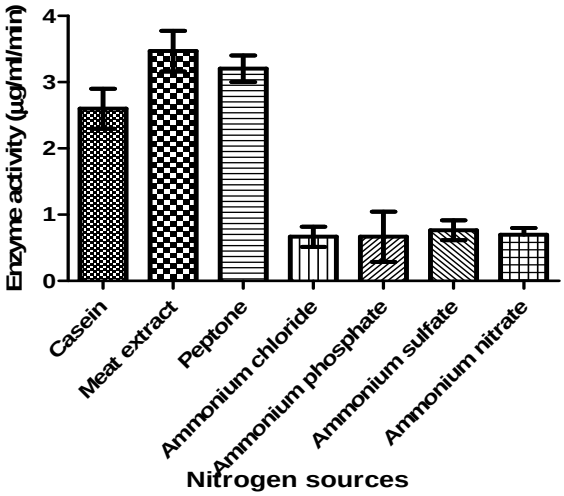

Fig. 7: Effect of incubation period on the activity of alkaline protease from *Comomonas kerstersii* KSM7

Partial purification and SDS-PAGE analysis

In the present study, partial purification of protease enzyme was performed by ammonium sulphate precipitation followed by dialysis. Partially purified protease with molecular weight of ~25 kDa was observed in SDS-PAGE (Fig 8). Similarly in *Bacillus cereus*, Abou-Elela *et al*. (2011) have reported the molecular mass determined using SDS-PAGE, was nearly 31.0 39 kDa [31]. Also Usharani and Muthuraj (2010) reported the protein of 15 kDa in *Bacillus laterosporus* [32].

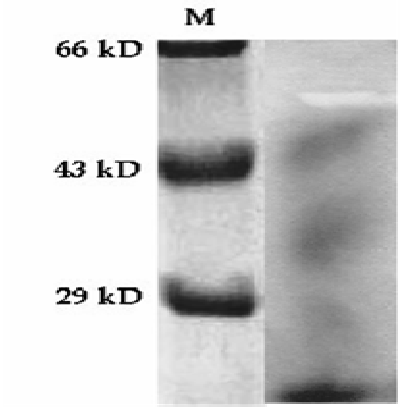

Fig. 8: The SDS-PAGE analysis of partially purified alkaline protease.

CONCLUSION

From the results, it has been concluded that the newly isolated *Comomonas kerstersii* KSM7 from soil sample can be considered as a possible candidate for large scale production of extracellular proteases. The best incubation period was found to be 72 hrs. Screening of suitable medium ingredients plays an important role in the production of proteolytic enzymes by *Comomonas kerstersii* KSM7. The optimum temperature and pH were detrermined as 60^0C and 9.0 and the best carbon and nitrogen sources were glucose and meat extract. Further the production process can be commercialized after further optimization for enhanced enzyme production and characterization.

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