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Original Article

Isolation, partial purification, biochemical characterization and detergent compatibility of alkaline protease produced by *Bacillus subtilis*, *Alcaligenes faecalis* and *Pseudomonas aeruginosa* obtained from sea water samples

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ABSTRACT

In the current study, bacteria isolated from sea water samples of Murdeshwar, Karnataka, were screened for the production of alkaline protease by culturing them onto skim milk agar media. Of the isolated bacteria, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Alcaligenes faecalis* showed distinct zones of hydrolysis due to enzyme production. They were each inoculated into enzyme production media under submerged fermentation conditions at 37 °C for 48 h with a constant agitation of 120 rpm. Partial purification of alkaline protease was carried out by isoelectric precipitation. Enzyme activity was determined under varying conditions of pH, incubation temperature, different substrates, carbon and nitrogen sources and salt concentrations using sigma's universal protease activity assay. Enzyme immobilization was carried out using 2% Sodium alginate and 0.1 M ice cold $CaCl_2$ and its activity under varying pH, temperature conditions and detergent compatibility was assayed. Efficacy of enzyme in stain removal was tested and haemolysis was observed within of 60 s which resulted in removal of the stain. Among the three organisms, enzyme from *Bacillus subtilis* showed highest activity in all cases indicating that it was the most ideal organism for enzyme production.

1. Introduction

Detergents are very important in fabric cleaning process because they disperse well in water, do not damage the fabric or our body on exposure and cleanse different types of stains. Detergents can clean stains of protein, chemical, fat, carbohydrate or any other origin. A Laundry detergent formulation contains a multitude of components and comes in solid powder form to liquid formulations [1]. A typical laundry detergent powder, contains the following components: Builders such as Sodium tripolyphosphate (Inorganic), nitrilotriacetic acid (Organic) or polycarboxylates (Polymer) to eliminate hardness and maintain alkalinity, anionic, cationic or non-ionic surfactants, bleaching agents like percarbonates or perborates and lastly additives like enzymes to make the cleansing process more efficient and perfuming agents to impart fragrance [2,3]. Detergents remove stains by electrostatic interactions and steric hindrance. The negatively charged micelles of detergent bind to the dirt particles and also to fabric and imparts a negative charge to them. Hence in presence of water, these micelles repel each other by electrostatic repulsions which prevent dirt particles from sticking to the fabric surface again. These micelles are then washed away [4]. Enzymes constitute an important part of detergent formulations. Enzymes are highly beneficial because they reduce activation energy of a reaction thereby making a reaction process more efficient with reduced energy consumption. By optimizing parameters we can ensure that enzymes function most efficiently and deliver the desired. The various parameters that can be optimized are pH, temperature of reaction, dosage, etc. The use of enzymes in chemical processes saves substantial amount of water [5]. Enzymes find application in various industries such as tanning, pharma, brewery, dairy, detergent and many more. Particularly in the detergent industry, the types of enzymes used in detergent formulations range from lipases, amylases, cellulases to proteases. Proteases are group of enzymes

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Table 1
Biochemical tests used to identify bacteria.

Tests	*Bacillus subtilis*	*Pseudomonas aeruginosa*	*Alcaligenes faecalis*	*Escherichia coli*	*Shigella flexneri*
Gram staining	Gram positive rods	Gram negative Rods	Gram negative coccobacilli	Gram negative Rods	Gram negative rods
Catalase	+	+	+	+	+
Oxidase	–	+	+	–	–
Indole	–	–	–	+	–
Methyl Red	–	–	–	+	+
Voges-Proskauer	+	–	–	–	–
Citrate	+	+	+	–	–
Urease	–	+	–	–	–
TSI agar (Slant/Butt)	Red/Yellow	Red/Red	Red/Red	Yellow/Yellow	Red/Yellow
Glucose fermentation	+	–	–	+	+
Lactose fermentation	–	–	–	+	–
H_2S production	–	–	–	–	–
Starch hydrolysis	+	–	–	–	–
Gelatin liquefaction	+	+	–	–	–
Nitrate reduction	+	Denitrifying	–	+	+

that bring about the hydrolysis of peptide bonds. Depending on the type of protease involved, peptide bonds adjacent to specific amino acids are cleaved. Among different proteases, alkaline protease has gained utmost utility in the detergent industry as it functions optimally above pH 8 and detergent formulations also have very high pH ranging from 9.0 to 12.0. Microorganisms serve as an important source of this enzyme in contrast to neutral proteases (with optimum activity around pH 7), which are mainly of plant origin [6]. Enzymes could be used as dry lyophilized beads in detergent powder formulations or they could be used as liquid enzymes. However, stability of liquid enzymes is an important concern and they are used as insolubilized enzymes in dispersed formulas, such as liquid dispersion products (LDPs) which contain components such as polyols, sugars, organic acids, metal salts, and water, to balance enzymatic stability. Loss in activity of enzymes could not only be caused by proteolysis but also by oxidation, or degradation by other chemical components of detergent or even by other enzymes. Therefore, enzymes must be highly tolerant to proteolysis and must have a long shelf life [7]. In the current study as well, determination of most optimum conditions for production of alkaline protease from halophilic *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Alcaligenes faecalis* is done and application of this enzyme in detergent formulation has been focused on.

Fig. 1. Casein hydrolysis seen as zone of clearance around colonies on skim milk agar.

2. Materials and methods

2.1. Isolation and biochemical characterization of bacteria from sea water sample

Sea water sample (undiluted) was subjected to serial dilution [8]. Five distinct bacterial colonies were obtained after incubation and identified by biochemical characterization and maintained as pure cultures [9,10]. Table 1 lists all biochemical tests conducted.

2.2. Screening for alkaline protease producing bacteria

Each organism was screened for production of extracellular alkaline protease by inoculation onto skim milk agar with some modification which consisted of Skim milk powder – 5%, Peptone – 0.25%, Yeast extract – 0.5%, Glucose – 1%, Agar – 2.5% and pH 8 and incubated at 37 °C for 24 h [11]. Skim milk powder and glucose was however sterilized separately and then added to autoclaved medium. It was observed that only colonies of *Bacillus subtilis*, *Alcaligenes faecalis*, *Pseudomonas aeruginosa* showed distinct zones of hydrolysis around them while *E. coli, Shigella flexneri* showed no zone of casein hydrolysis. Fig. 1 shows casein hydrolysis by *Alcaligenes* on skim milk agar.

2.3. Cultivation of bacteria in enzyme production media

Loop full of cultures of *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Alcaligenes faecalis* were inoculated into separate flasks containing alkaline protease production media which was made up of the components of Horikoshi-I alkaline medium (pH 9) i.e. D-glucose-10 g, Peptone-5 g, Yeast extract-5 g, KH_2PO_4-1 g, $MgSO_4 \cdot 7\ H_2O$-0.2 g, Na_2CO_3-5 g and Distilled water-1000 ml [12]. Glucose and sodium carbonate were autoclaved separately and then added to rest of the autoclaved medium and incubated at 37 °C for 48 h with continuous agitation of 120 rpm. After 48 h, matt growth of bacteria was seen in the liquid media. The media was filtered using Whatman® filter paper in order to remove the microbial matt. The filtrate so obtained was further centrifuged at 4000 rpm for 10 min to remove any residual particles that may be present in it. The filtrate was then separated and used for further process [13].

2.4. Separation and partial purification of alkaline protease by isoelectric precipitation

The filtrate obtained after centrifugation was subjected to isoelectric precipitation in order to obtain crude alkaline protease

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