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Optimization of Culture Growth Parameters for Production of Protease from Bacteria, Isolated from Soil

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Abstract

Proteases are multipurpose group of enzymes used in various industries such as detergent, silver recovery, food, pharmaceutical, leather, and textile industries. This work aimed to produce protease from local microflora for use as detergent additive and silver recovery from waste X-ray film. Isolation of protease producer undertaken using skim milk agar medium and process parameters was optimized. Crude enzyme was characterized followed by stain and gelatine removal tests. A total of 22 protease positive bacteria were isolated from the study area. Out of 22 protease producing bacteria, one isolate designated as Bacillus sp. CAMA14 was selected. Maximum protease production was achieved at 2.5% (v/v) inoculums size, 1% (w/v) NaCl, pH 8.0 at 30°C and 48 hr. Glucose and yeast extract were best carbon and nitrogen sources respectively. The optimum activity was reached at pH 9 and 50°C. The enzyme was stable in the pH range of 7 to 10. It retained 75%, 86% and 72% activity after one hr pre-incubation at 50°C, in 15% H_2O_2 and 0.3% commercial detergent respectively. The enzyme activity was increased by Mg^{2+}, Cu^{2+}, and Mn^{2+}, was not affected by Ca^{2+} but decreased by Zn^{2+}, Hg^{2+} and Fe^{2+}. It removed stains of egg yolk on cotton cloth, and gelatin on X-ray film at pH 9, 40°C, in 40-46 min. These properties suggest that protease from Bacillus sp. CAMA14 could find potential application in detergent industries and silver recovery process.

Keywords

Bacillus Sp. CAMA14, Detergent, Gelatine, Protease, X-ray Films

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1. Introduction

Proteases are essential constituents of all form of life on earth including prokaryotes, fungi, plants and animals. Although, animal and plant proteases are of important industrial applications, a large proportion of commercially useful proteolytic enzymes currently available in the market are from microorganisms. Proteolytic enzymes from microbial sources are preferred over the enzymes derived from plant and animals since they possess almost all characteristics desired for their biotechnological applications [1]. In addition microbial proteases represent one of the largest groups of industrial enzymes and account for approximately 60% of the total industrial enzyme sale in the world. Considering this market demand, there is a need to effort to solve this problem by investigating new microorganisms because they are the major sources of all commercially important alkaline proteases, which have unlimited industrial applications.

One area of well-established application of biotechnology is the use of enzymes in detergents [2]. Enzymes have long been of interest to the detergent industry for their ability to aid in the removal of proteinaceous stain such as milk, blood, egg, meat and fish and protein from body secretion and to deliver unique benefits that cannot otherwise be obtained with conventional detergent technologies. Therefore, addition of proteases in the detergent helps to degrade the protein and

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greatly improve the washing performance of standard detergents. Due to potential usefulness of alkaline thermostable protease in bio-detergent industry, the development of methods for cheaper production of enzymes is very important.

Most commercially available detergents have an alkaline pH and contain chelating agents to overcome water hardness. As result enzyme used for detergent application need to be active and stable at alkaline pH, ionic strength of detergent solution, different washing temperature range (25-60°C). There are also many more parameters involved in the selection of a good detergent protease, such as compatibility with detergent components, e.g. surfactants, perfumes and bleaches; stain degradation and shelf life [3, 4]. Good detergent enzyme should also be stable in the presence of oxidizing agents and bleaches.

Another area of application for proteases is for recovery of silver from waste X-ray film. The conventional method for recovery of silver is burning and chemical treatment of the films directly. This generates undesirable foul smell and environmental pollution. In addition polyester film on which emulsion of silver and gelatin is coated cannot be recovered. But recently, microbial proteases are being use as alternatives to the burning and chemical methods of silver recovery from X-ray films. This not only helps in extracting silver, but also the polyester film base can be recycled. Alkaline protease decomposed the gelatin layer and releases the silver [5, 6]. Bearing in mind this critical factor, effort should be directed to search microorganisms which produce proteases with high stability in the environment of silver recovery process.

Besides to detergent formulation and silver recovery, proteases are useful in dehairing for the purpose of leather manufacture. Enzyme based dehairing processes using proteases preferred over chemical method due to enormous environmental benefits [3]. However most of leather manufacturer not used the protease for dehairing purpose due to cost of substrate for protease production. To tackle this critical factor, effort should be directed to production of such valuable enzyme using cheap and readily available growth substrate.

Microorganisms have still potential to produce enzymes, even though production cost of the enzyme is the critical issue for further application at industrial level. Substrates from agro-industrial wastes such as Banana peel, cow dung, sugar cane biogases, Coconut cake, Groundnut and so on are cheap and readily available growth substrates [7]. Therefore, the feasibility of enzyme production on low cost fermentable substrates needs to be studied. These greatly reduce the production cost beside the removal of these undesirable wastes which indirectly minimize environmental pollution.

Many researchers in Ethiopia have been engaged in many research aspects of bacterial species. But, few of the researchers have been tried to isolate, optimize and characterize specific bacterial species for production of protease and no one evaluate its application for detergent industry and recovery of silver. As result there are relatively no reports that support the scientific literature on this topic from Ethiopia. Therefore a research project has been initiated to investigate protease from bacteria isolated from locally available bacteria species and examine their potential to be used as detergent additives and silver recovery and to generate information to support the scientific literature.

2. Materials and Methods

2.1. Sample Collection and Isolation of Proteolytic Bacteria

Samples were collected from Arba Minch town Compost processing site. Each sample was kept in clean sterile sample bottles sealed and transferred to the microbial biotechnology laboratory and stored at 70C.

The soil and wastewater samples were suspended in water by vigorous vortexing and serial dilutions were made and appropriate dilution were added to petri plate on skim milk agar plate containing peptone (0.1%), NaCl (0.5%), Agar (2%) and skimmed milk (10%) at pH 9 (the pH adjusted by adding around 1% Na_2CO_3 after autoclaving both separately) and incubated at 400C for three days. Alkaliphilic proteolytic bacteria were screened. A clear zone formation around the colonies due to skim milk hydrolysis indicated alkaline protease production by the microbes [8]. These colonies were picked and purified by streaking on skim milk agar. The cultures were subsequently sub-cultured and used regularly. Agar slants were prepared and preserved at 7°C for further experiments and in 25% glycerol stocks at -25°C for long term storage.

2.2. Screening and Characterization of Potential Isolate

The bacterial strains were screened for the quality of enzyme produced under submerged condition. The supernatant from each isolate was used as crude enzyme for observing individual wash performance and gelatin removal efficiency. Single isolate which have high potential on egg stain removal from cloths and gelatin removal from used X-ray film was selected for further study. Finally various biochemical studies were undertaken according to Bergey's manual of determinative bacteriology for identification of the selected potential isolate [9].

2.3. Protease Assay

Protease production media containing (g/l): Glucose 10, Peptone 7.5, $MgSO_47H_2O$ 5, K_2HPO_4 2 and $CaCl_2$ 1 [10] was

prepared for production. The bacterial isolate was inoculated with 1% of inoculums into production medium followed by incubation at 30°C for 48 hours in rotary shaker at 150 rpm. Culture filtrates were separated by centrifugation at 5000 rpm for 15 min and the supernatants were used as crude enzyme source for quantitative enzyme assay.

Protease activity in the culture supernatant was determined using casein as a substrate with some modification of the method described by Gaur et al. (2010) [11]. The enzyme activity was expressed in units (U) and calculated by using tyrosine standard calibration curve. One unit of enzyme was defined as the amount of enzyme that releases 1 µmol of tyrosine per ml of crude extract per minute under standard conditions at 40°C. The tyrosine standard curve was used to generate the enzyme unit determination formula in U/ml.

2.4. Optimization of Culture Condition

Various process parameters influencing enzyme production: incubation time (0-96 hrs.), pH (7-11) [12], inoculum size (1-10%) [13], salt concentration (0-20%), various carbon sources (5.0% w/v) and nitrogen sources (0.5% (w/v) [8] were optimized. To find out the suitability of agro based wastes as substrate for enzyme production banana peels, cow dung, sugar bagasse, corn con and cassava peels were taken in the growth medium under submerged condition [14].

2.5. Crude Enzyme Characterization and Compatability

The protease activity of Bacillus sp. CAMA14 at different pH values was tested with 0.005M sodium bicarbonate buffer (pH 7.0 to 10.0) and 0.02M potassium chloride/NaOH buffer (pH 11.0 to 12.0). pH stability was determined by preincubating the crude supernatant in buffers of pH (7-12) at room temperature for 1 hr. The enzyme activity was measured at 40°C. To determine thermal stability, the samples were pre-incubated at 50°C following determination of residual activity every 20 minutes. Relative protease activity (expressed in%) was defined as the percent protease activity compared with the maximum value.

To investigate the effect of oxidizing agents on enzyme stability, hydrogen peroxide was used from 0-30% in the reaction mixture. Pre-incubation of the reaction mixtures was carried out by mixing equal amount of enzyme and H_2O_2 [6].

A commercial powder detergent, Aerial was used for detergent compatibility test of enzyme produced by Bacillus sp. CAMA14. The detergent was heated at 100°C for 1hr to denature all enzymes found in the detergent [6]. The enzyme from Bacillus sp. TF44 was preincubated with 0.3% (w/v) heated detergent for 1 hr and residual activity was measured to check the compatibility of the enzyme with all the surfactants found in the detergent. Enzyme without detergent was used as a control.

2.6. Washing Test with Protease Preparation

Washing performance of crude enzyme was determined according to the method of Pathak and Deshmukh (2012) [6] with little modification.

2.7. Removal of Gelatinous Coating from Used X-ray Film

Removal of gelatin from X-Ray film was done as described in Shankar et al. (2010) [15] with some modification.

2.8. Experimental Design and Statistical Analysis

Culture conditions: incubation time, pH, inoculum size, salt concentration, and carbon and nitrogen sources selected as main influencing factors in the protease production were optimized by one factor at a time method. Average values of duplicate experiments were taken. Microsoft office Excel worksheet 2010 was used for data analysis and presentation.

3. Results

3.1. Isolation and Screening of Potential Isolates

Twenty two (22) protease positive isolates were obtained from the sample area (compost processing site). Hence the strains were identified as a protease producer (Figure 1). Out of 22 protease positive bacterial isolates, 5 isolates with relatively higher clear zones were further examined.

Figure 1. Zone of hydrolysis by strain CAMA14 in milk agar plate after 18 h incubation at 40°C.

Among the 5 isolates, one isolate was selected on the basis of egg yolk stain removal performance from cloths and gelatin removal efficiency from waste X-ray film. Accordingly, isolate CAMA14 was selected for further study.

3.2. Morphological and Biochemical Characteristics of Isolate CAMA14

Results of morphological and biochemical tests of the selected isolate (CAMA14) are; the isolate was characterized as Gram positive and catalase and motility positive. The colonies were characterized as opaque, irregular and spready configuration and irregular margin. Motile, long irregular, elliptical endospore and rod shaped cells were observed under light microscope with 1000X magnification. The strain was able to hydrolyse casein and indole positive. The result of nitrate reduction, citrate utilization and MR-VP test were been negative. Based on this the isolate was designated as Bacillus sp. CAMA14 (Figure 2).

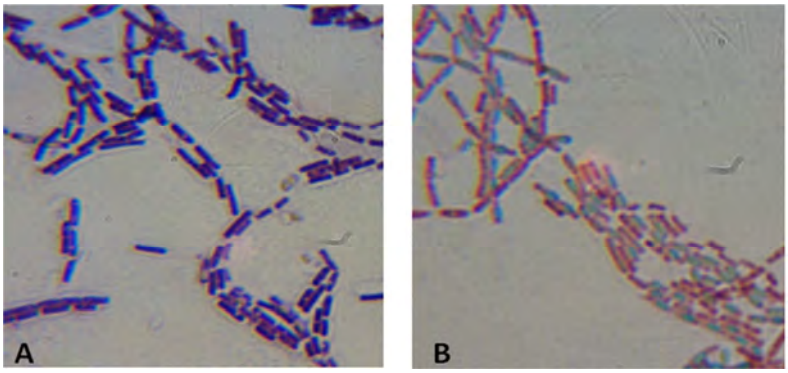

Figure 2. Gram reaction result (+ve) (a) and Endospore of Bacillus sp. CAMA14 (b) at 40°C and 24 h on milk agar.

3.3. Optimization of Process Parameters for Enzyme Production

3.3.1. Effect of Incubation Time

As shown in Figure 3, the enzyme was gradually produced at the initial growth phase. The production was increased exponentially from 12 to 48 h. Maximum enzyme production was observed at 48 h of incubation. The production was gradually decreased after 48 h.

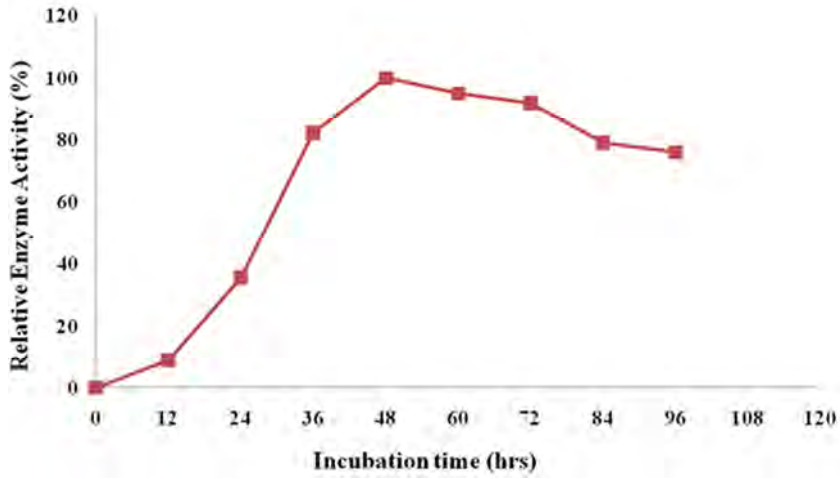

Figure 3. Effect of incubation time on enzyme production by Bacillus sp. CAMA14 at 30°C, 150 rpm and pH 9

3.3.2. Effect of pH

The effect of pH on protease production is shown in Figure 4. The maximum protease production was obtained at pH 8.0. Minimum amount of protease production (19% of its maximum production) was recorded at pH 11. Protease production declined sharply after pH 9.

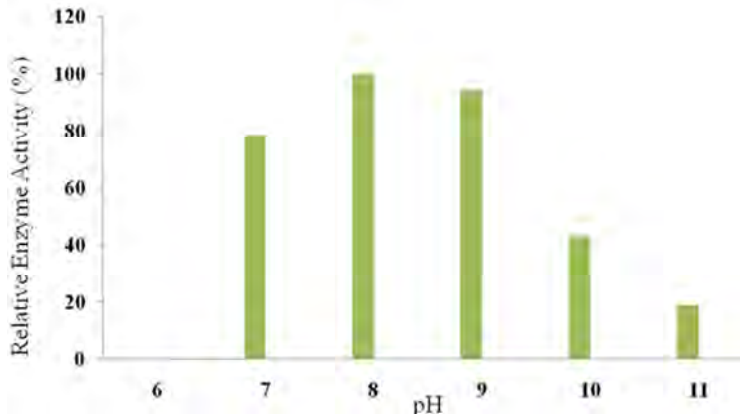

Figure 4. Effect of pH on enzyme production at 30°C and 150 rpm for 48 h.

3.3.3. Effect of Inoculum Size

In order to determine the effect of inoculum size on protease production of Bacillus sp. CAMA14, the production medium was inoculated with 1 to 10% of a 24 h culture in 2.5 intervals. The optimum inoculum size for protease production was 2.5%. The isolate expressed 49 and 28.73% of its maximum production at 7.5% and 10% inoculum sizes respectively (Figure 5).

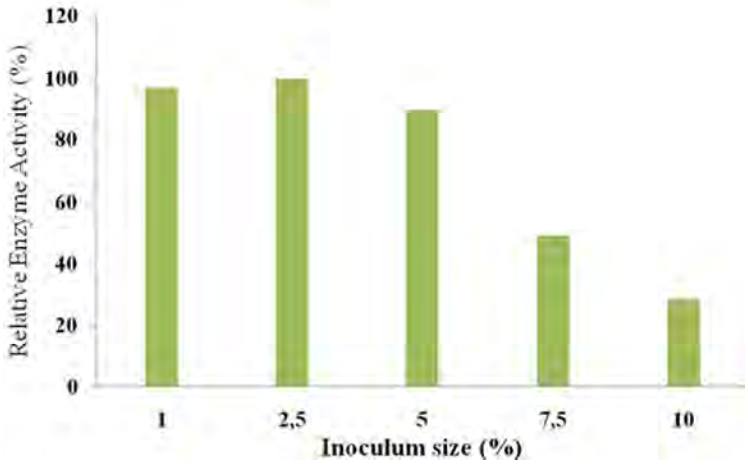

Figure 5. Effect of inoculum size on enzyme production after 48 h incubation at 30°C, 150 rpm and pH 8.

3.3.4. Effect of Carbon Sources

Various carbon sources lactose, sucrose, glucose, mannitol, starch and maltose were used to study their effect on the enzyme production. Glucose and mannitol caused the maximum and minimum productions respectively. The results are shown in Figure 6.

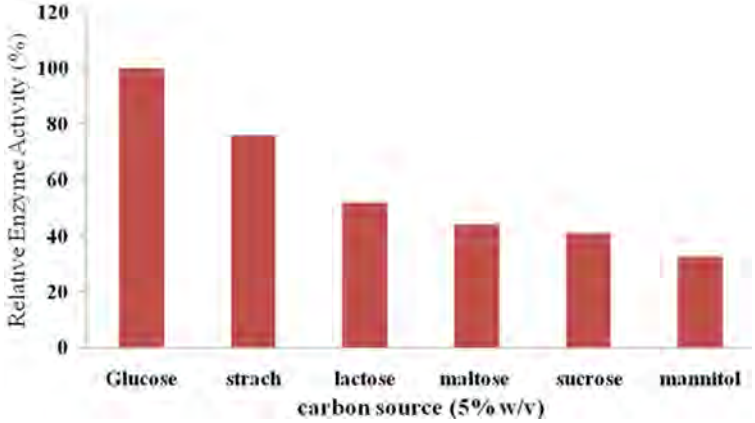

Figure 6. Effect of carbon sources on enzyme production at 30°C, 150 rpm and pH 8 for 48 h.

3.3.5. Effect of Nitrogen Sources

The comparative fermentation trial results with five different nitrogen sources are indicated in Figure 7. The highest enzyme activity was attained with yeast extract whereas the minimum level of enzyme production was achieved with urea.

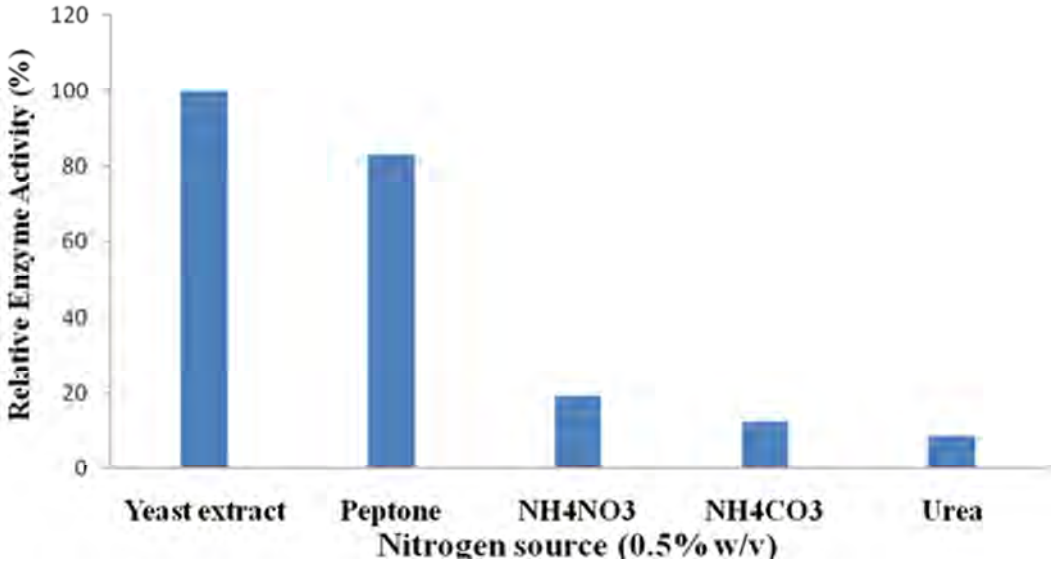

Figure 7. Effect of nitrogen sources on enzyme production at 30°C 150 rpm and pH 8 for 48 h.

3.3.6. Effect of Salt Concentration

Among the different concentrations of NaCl tried for enzyme production the highest production was obtained at 1% NaCl. As the NaCl concentration increased enzyme production declined consistently (Figure 8).

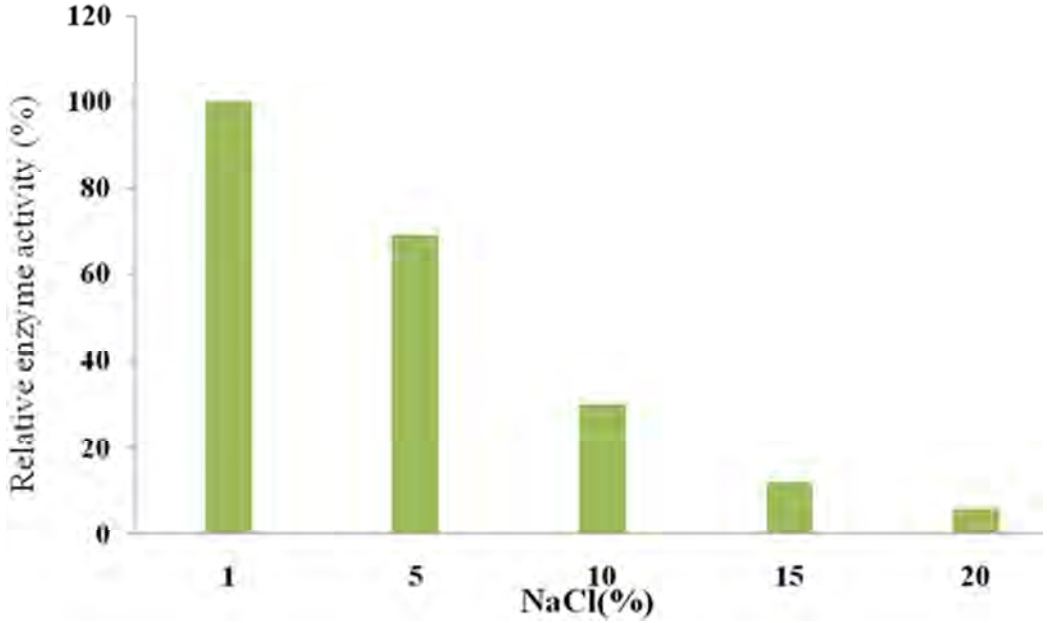

Figure 8. Effect of salt concentration on enzyme production at 30°C, 150 rpm and pH 8 for 48 h.

3.3.7. Production Optimization using Agricultural Residues

From the various agricultural wastes used for the cheap production of protease by Bacillus sp. CAMA14, the maximum enzyme production was achieved in sugarcane bagasse. Comparable results were obtained from Corn con and banana peel. Cassava peel resulted in the least enzyme production. However all the tested substrates had promising production levels (Figure 9).

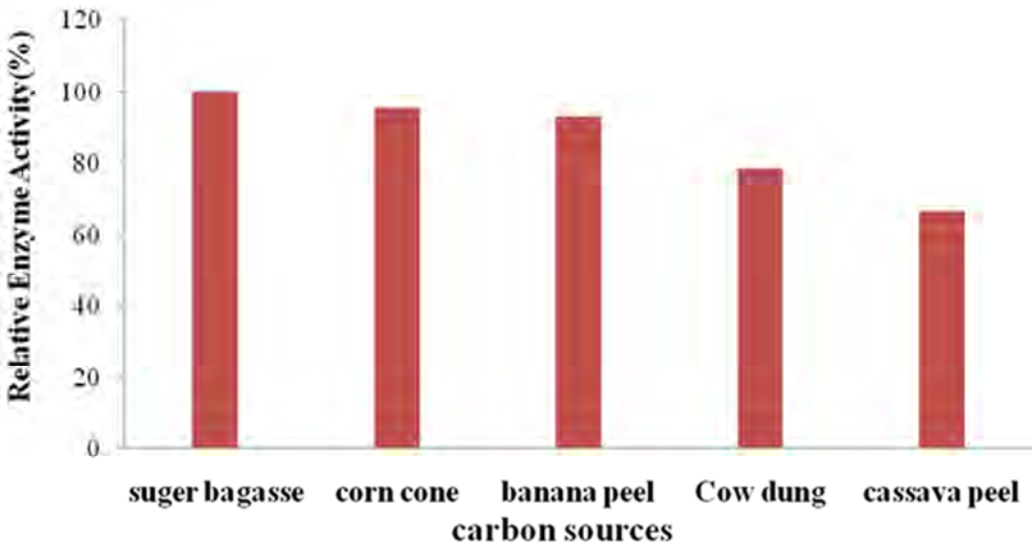

Figure 9. Enzyme production potential of some agricultural residues at 30°C, 150 rpm and pH 8 for 48 h under submerged condition.

3.4. Stain Removal Efficiency of Crude Enzyme

The crude enzyme produced from Bacillus sp. CAMA14 efficiently removed egg yolk stain in 40 min (Figure 10).

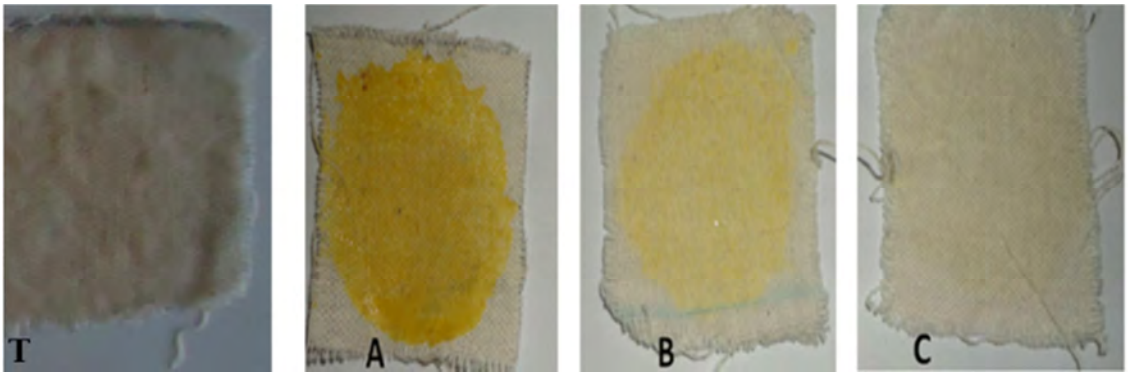

Figure 10. Egg yolk stain removal efficiency of enzyme produced by Bacillus Sp. CAMA14: (T) normal cotton Cloth; (A) Stained cotton fabric before reaction mix; (B) control (0% of enzyme/ only phosphate buffer); (C) with 5.07U/ml of crude enzyme for 40 min at 40°C and pH 8.

3.5. Removal of Gelatinous Coating from Used X-ray Film

Complete gelatin removal from used X-ray film was observed at pH 8 within 46 min at pH 8 (Figure 11).

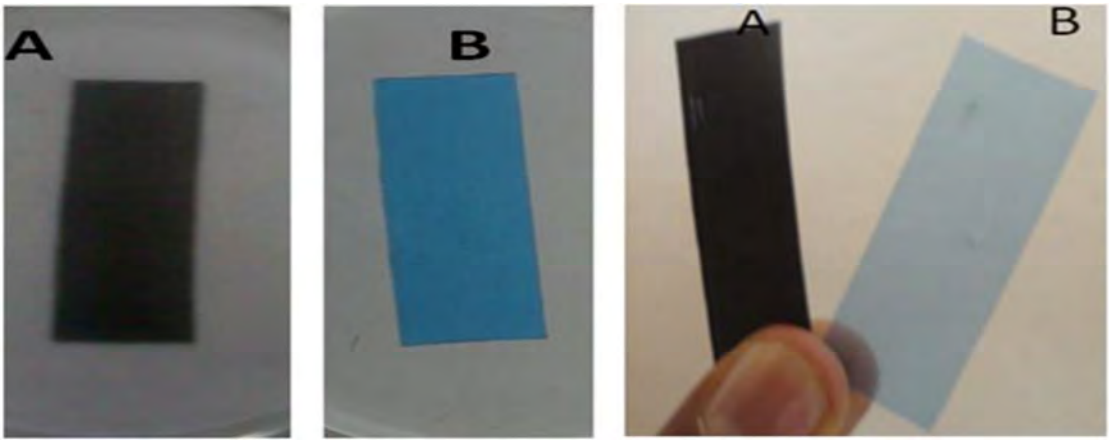

Figure 11. Hydrolysis of used X-ray film by crude enzyme produced from Bacillus sp. TF44. Sample pictures: (A) control (20ml of Bicarbonate buffer) and (B) after gelatin layer removal from the film after 46 min incubation at 40°C and pH 8 with crude enzyme.

4. Discussion

One of the main concerns of this study was to isolate and optimize alkalophilic isolate having a vital ability to secrete extra-cellular proteolytic enzyme to be used for detergent additive and silver recovery. Accordingly, 22 bacterial strains were isolated from the study area. Formation of clear zone around the bacterial colony indicated the protease positive strains hydrolysed the skim milk present in the media (Figure 1). Out of this only 5 isolates were screened based on the size of clear zone. The use of skim milk agar for the isolation of protease producing bacteria has earlier been reported by some workers [8, 16].

Understanding of cell growth period in relation to enzyme production could be important factor for identifying the appropriate incubation period for maximal enzyme productivity. In this study, highest enzyme production was observed after the Bacillus sp. CAMA14 reached around 48 h of cell growth. Periods before and after 48 h were not the convenient harvesting time for protease produced from Bacillus sp. CAMA14. For instance, after 48 h of incubation a decrease of enzyme production was observed (Figure 3). This may occur due to reduced microbial growth and increment of cell death, which is associated with the depletion of available resource, required for the growth of Bacillus sp. CAMA14 cells. These results were synonymous to that has been investigated by many researchers [17, 18, 19].

Initial pH of production medium is another important factor that significantly influences production of protease. It has been noted that microorganisms are dependent on the extra cellular pH for their cell growth and enzyme production [17]. As shown in Figure 4, maximum enzyme production was achieved in a medium of pH 8 which was the optimum pH for production of enzyme from Bacillus sp. CAMA14. Similar result was obtained by Padmapriya et al. (2012) [1] in production and purification of protease from Marine Bacillus Sp. and by Geethanjah and Subash (2011) [21] in optimization of protease production by Bacillus subtilis. Also in agreement to this result [18, 22, 23] have the same reports that pH 8 is optimum for maximum enzyme production.

The appropriate inoculum size is an important factor for protease production. As shown in Figure 5, maximum protease production was achieved at 2.5% inoculum size, which corresponded to 24 hr inoculum age, which is the best inoculum size for enzyme production from the strain. At lower inoculum size the isolate may have extended lag phase that result in insufficient number of microorganisms for production of required enzyme [24]. At inoculum size above the optimum, the microorganisms may face unfavourable condition like that of depletion of nutrients, change in availability of oxygen, and competition to access limited resources to result low protease production [4]. These results are similar with Elibol et al. (2005) and Kalaiarasi and Sunitha (2009) who reported that 2.5% inoculum level gave higher protease production [22, 25].

Requirement for specific carbon and nitrogen source differs from organism to organism, or even among the same species isolated from different sources [20]. Glucose is the best carbon source for high protease production from Bacillus sp. CAMA14 (Figure 6). This finding was in accordance with well documented reports in literature for protease production [21, 26].

Another nutritive factor influencing protease production is the nitrogen sources. It is also well documented in the literature that nitrogen is metabolized to produce primarily amino acid, nucleic acid, and protein and cell wall components. These nitrogen sources have regulatory effect on the enzyme. It was found that the optimum enzyme production had been established in case of yeast extract. Peptone could also be good nitrogen source in the absence of yeast extract (Figure 7). This implies existence of close relationship in the type of nitrogen sources used as a supplement and amount of protease production [18, 27, 28].

The influence of NaCl on enzyme productivity was determined. NaCl concentration of 1% w/v was found to be ideal for optimum enzyme yield from Bacillus sp. CAMA14. As shown in Figure 8, increase in salt concentration resulted in sharp decline of enzyme production. This implies that Bacillus sp. CAMA14 was sensitive for salt concentration in production medium. So determining the appropriate salt concentration is crucial for maximal enzyme production. Several reports are available with respect to NaCl tolerance in the literature and consistent with this result [17, 18, 23].

In the present investigation (Figure 10) the protease produced from Bacillus sp. CAMA14 had high capability of removing blood and egg yolk stains from cloths. Therefore, protease produced from Bacillus sp. CAMA14 could be one of the most alternative detergent additives. The result is far better comparable to the earlier reports of protease from Pseudomonas aeruginosa [29], Bacillus licheniformis U1 [30] and Bacillus sp. Cab44 [31].

As shown in Figure 11, used X-Ray films treated with protease produced from Bacillus sp. CAMA14 resulted in the silver bound with gelatin being stripped off in to the reaction mixture after 30 min at pH (9) and 40°C. This implies protease produced in the present study has appreciable application in the process of silver recovery. Similar results were reported by many researchers [32, 33, 34, 35].

5. Conclusions

Based on the results obtained from this study, the following conclusions could be drawn:

A total of 22 protease producers were isolated and out of this, one selected as best. Maximum enzyme was obtained from this strain at pH 8, 2.5% inoculum size, 1% NaCl and after 48hrs incubation time. Such strain also showed maximum enzyme production using glucose and yeast extract as carbon and nitrogen source respectively. The enzyme produced in this investigation is highly efficient for proteins stain removal from cloths. All these properties deduce that its suitable application as detergent additive for formulations of various detergents.

The enzyme also has a potential to remove gelatin layer from used X-ray film so that it is applicable in silver recovery process. In addition production of the protease by using cheap and readily available substrates offer an advantage in minimizing production cost to scale up production for its use in various industries.

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