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Research Article

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Production of Alkaline Protease from Dairy Sludge and its Application as Stain Buster

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ABSTRACT

Proteolytic bacteria are most important for industries such as food and fermentation thus isolating and manipulating pure cultures from various source has important for various biotechnology industries Different varieties of bacterial species (SB1,SB2,SB3,SB4)are isolated from "Dairy sludge" and all the isolates will be screened for their proteolytic activity using skimmed milk agar plates, in which SB3 are found to be protease producing lactobacilli .Production of alkaline protease is carried out in modified production medium , centrifuged and supernatant was taken as crude enzyme. Industrial applications as stain buster ,as it used for the removal of blood stain which shows the maximum removal of stains by its proteolytic activity.

Keywords: *Dairy sludge, skim milk agar, alkaline protease, stain buster.*

INTRODUCTION

Proteases are the most important industrial enzymes that execute a wide variety of functions and have various important biotechnological applications[1]. They constitute two-thirds of the total enzymes used in various industries and account for at least a quarter of the total global enzyme production[2]. Among all the most common types of enzyme known to us, proteases is one of the most essential and important enzyme having a major role in industrial and biotechnological applications. It has been used in a wide range of industries and its use account for about to third of all the enzymes used[3,4]. Alkaline proteases produced are of special interest as they could be used in manufacture of detergents, food, pharmaceuticals and leather[5,6]. It is also widely produced globally. Various microbes such as bacteria, fungi and yeast along with many plants and mammalian tissues are capable of producing alkaline protease. In recent years the increasing demand for protease leads to the concept of some exotic microbial strains that are capable of producing protease which can be used as biocatalyst in various fields of biotechnology and industrial microbiology[7,8]. Production of alkaline protease is dependent on the nature of strain and the conditions for growth such as pH, temperature, nutritional requirements and incubation time. The production media should be cheap and it is known that *Bacillus* sp. is most efficient producer of alkaline protease[9]. Various species of *Bacillus* are used for the production of protease and such microbial proteases are of great importance due to its immense role in the formulation of detergents. So there is need to search for such new strains of Bacillus which has the ability to produce proteolytic enzyme with unique properties[10]. Keeping this in mind, our present study was carried out for the production of alkaline protease from dairy sludge and determining its efficiency as a stain buster.

MATERIALS AND METHODS

Sample Collection

Dairy sludge samples were collected from dairy industry at Vellore ,Tamil Nadu.

Isolation of Bacteria from dairy sludge

Collected dairy sludge sample was serially diluted (upto 10^{-6} dilution) and 0.1 ml of 10^{-5} and 10^{-6} were spreaded on nutrient agar medium and incubated at 37°C for 24 h. After 24 hrs incubation different

colonies were seen in nutrient agar plate colonies were studied and according to colony morphology 4 types of colonies (SB1,SB2,SB3,SB3) were selected for further process. After observing the colony morphology pure culture was prepared from SB3 strain.

Preliminary screening by Casein Hydrolysis

To determine whether an organism can produce the exoenzyme casesase.whose principle is that Caseine is an exoenzyme that is produced by some bacteria in order to degrade casein. Casein is a large protein that is responsible for the white color of milk. This test is conducted on milk agar which is a complex media containing casien, peptone and beef extract. Organism produce casein, and then there is be a zone of clearing around the bacterial growth . 100 µl of broth medium of pure culture was inoculated in the wells of skimmed milk agar plate by agar well diffusion method , and incubated at 37°C for 24 hrs.

Protease Production Medium

The production of protease was carried out in modified production medium[11]. 1 ml of the overnight grown bacterial broth medium was inoculated in 100 ml of modified medium containing (g/l): Dairy sludge-15, KH_2 PO $_4$-1, $MgSO_4$.$7H_2$ O-0.3, $FeSO_4$.$7H_2$ O-0.2, $ZnSO_4$.$7H_2$ O-0.2, $CaCO_3$ -1, pH-9.0.[12]. After incubation, at 37'C for 24 hrs medium was centrifuged at 10,000 rpm for 10 mins[13].

Estimation of protease activity

Pipette 1 ml of culture (SB3) extract "enzyme" into a test tube. Add 5 ml of .65 % casine in soluble phosphate buffer (0.1 M in ph-7.2). Add 1 ml of folin ciocolteau reagent rapidely and mixed properly.Incubate in water bath at 37ºc for 10 mins. Estimate protein content by using folin method[14].

Estimate of standard tyrosine

Stock solution:Dissolve 0.2 mg/ml(1.1mM) of tyrosine in distilled water (adjust the ph to alkaline). Pipette out various concentration of working standard solution into a series of test tubes and made up the volume to 1 ml with distilled water (100µ moles to 1000µ moles). Add 1 ml of diluted Folin-ciocolteas reagent rapidly and mix properly[15]. Incubate in water bath at 37ºc for 10 mins.

Enzymatic activity in blood stain removal

Four ml of blood was taken and kept in a test tube and lebelled as 100%.2ml of 100% blood sample was taken into 50% labelled tube and 2ml of deionized water was added into the tube labelled 50%.2ml of 50% solution was added into the 25% labelled test tube and 2ml of deionized water was taken into the test tube.2ml of 25% solution and 2ml 0f deionized water was taken into the 12.5% labelled test tube.2ml of 12.5% solution was discarded from the 12.5% labelled test tube.

5 pieces of white clothes was taken and labelled as given below

1.WATER- 12.5%.25%,50%.100%

2.CRUDE ENZYME-12.5%,25%,50%,100%

3.DETERGENT WITH CRUDE ENZYME-12.5%,25%,50%,100%

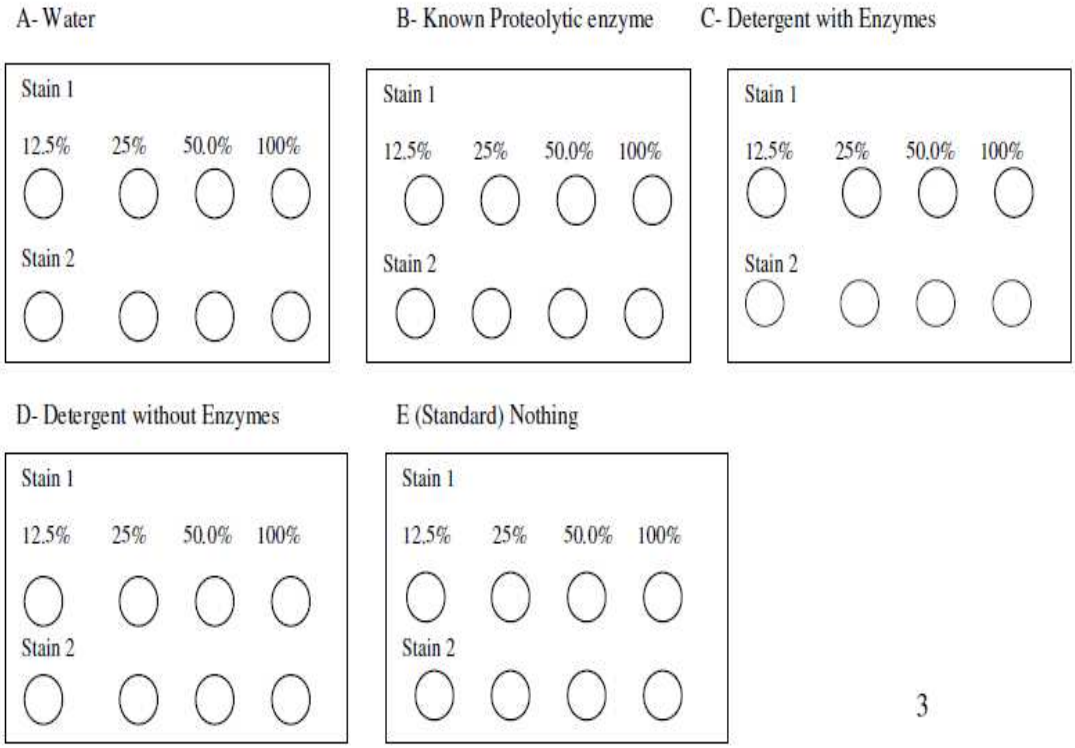
4.DETERGENT WITHOUT ENZYME-12.5%,25%,50%,100%

5.STANDARD-(NOTHING)-12.5%,25%,50%,100%

Respective concentrations of blood stain was dropped to all the five pieces of the white clothes.

The stains was allowed to air dry using drier.After the stains were completely dried,they were given the different treatments and kept for 5 to 10 mins and then rinsed with cold tap water.

The diagram below illustrates the experimental set up of the white fabric:



RESULT AND DISCUSSION

Isolation of Bacteria from dairy sludge

In the present research work four different types of bacteria were isolated from the dairy sludge and named as SB1 ,SB2,SB3,& SB4.the colony morphology of SB1 ,SB2 & SB4 was found to be circular and off white in colour ,whereas SB3 was found to be Irrigular in form & off white in colour.From gram staining all the SB1 ,SB2 & SB4 was found to be Gram negative rod shaped but SB3 was found to be Gram positive rod in single & pairs as given in Table 1,

Plate 1: pure culture from SB3 isolate

Plate 2: pure culture from SB3 isolate

The colonies were subjected to gram staining .The colonies which were positive for gram staining,subjected for further study.

Table 1: Colony morphology of isolated organism

Sample	Gram nature	Shape	Form	Colour
SB1	Negative	Rod chain	circular	Off white
SB2	Negative	Rod,chain	circular	Off white
SB3	Positive	Rod,single	irregular	Off white
SB4	Negative	Small rod	circular	Off white

Preliminary screening by casein hydrolysis

This test is conducted on skimmed milk agar which is a complex media containing casien, peptone and beef extract. Organism produce casein, and then there is be a zone of clearing around the bacterial growth .The crude protein extract from SB3 was subjected for casein hydrolysis in which it was determined that the SB3 stain lysed the casein protein as shown in the plate 3.

Plate 3: casein hydrolysis

The broth medium of pure culture that was inoculated in the wells of skimmed milk agar plate, showed clear zone around the wells.

Estimation of Protease activity

Protease are testing digests casein, the amino acid tyrosine is liberated along with other amino acids and peptide fragments. Folin & Ciocalteus Phenol, or Folin's reagent primarily reacts with free tyrosine to produce a blue colored chromophore, which is quantifiable and measured as an absorbance value on the spectrophotometer. The more tyrosine that is released from casein, the more the chromophores are generated and the stronger the activity of the protease.

Concentration of working SB3 crude enzyme was found to be 390 µg/ml at OD value 0.44 at 660nm of wavelengths. The protease activity was found to be units.

Calculation of protease activity

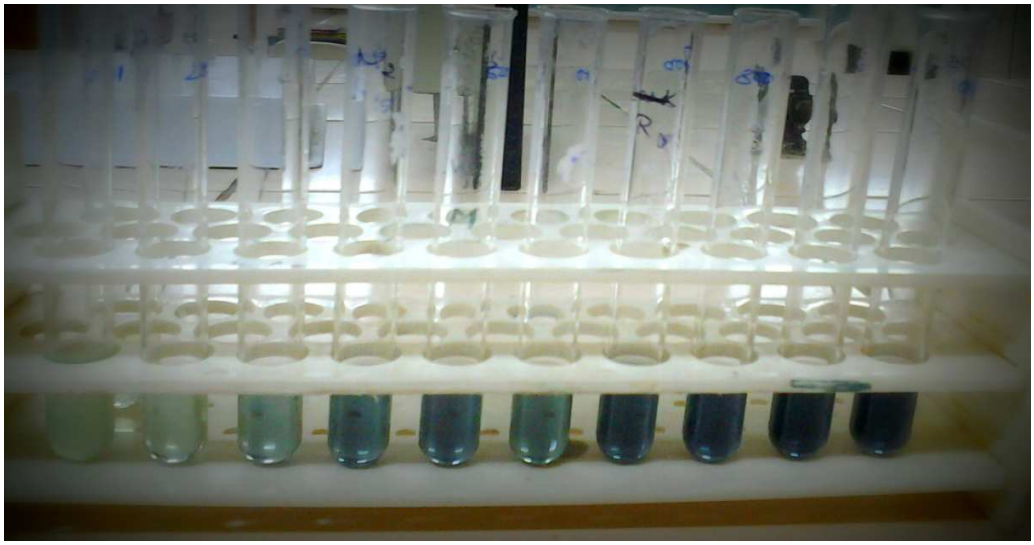
Au/ml:

(µ moles of tyrosine released × volume of sample) / (volume of enzyme used × incubation time × colorimetric volume)

= (390 × 4) / (1×10×2) units/ml

=78 units/ ml

Fig.1: Blue colour formation showing protease activity



Efficiency of crude Enzyme in stain removal

Today, one of the main applications of enzymes is in heavy-duty detergents for household laundry. The majority of enzymes used in laundry detergents are proteases for removing protein stains. We got the result after performing the application part as stain busters we observed that Sample with proteolytic enzyme shows the maximum activity in comparison to other samples(water,detergent+enzyme,detergent without enzyme), it breaks down the protein and remove the blood stain more efficiently on cloth as shown in the figure-4,5,6,7 respectively.

Fig. 2:

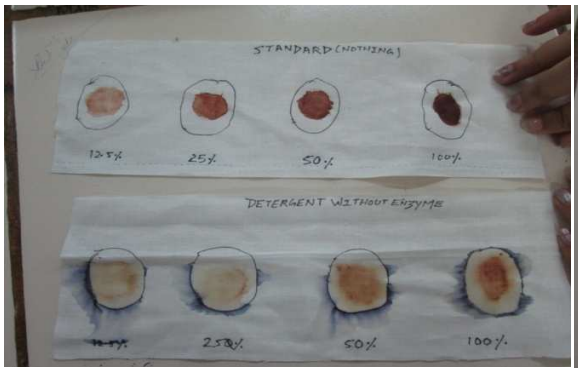


Fig.3:

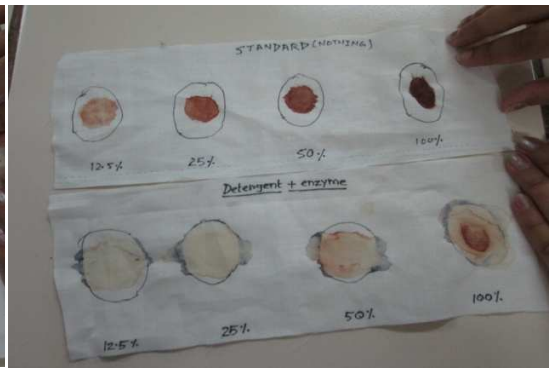


Fig.4:

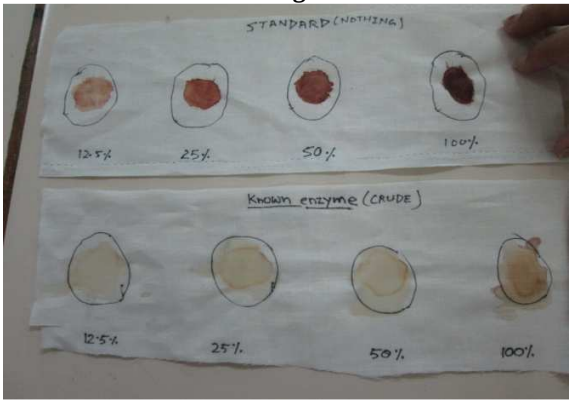
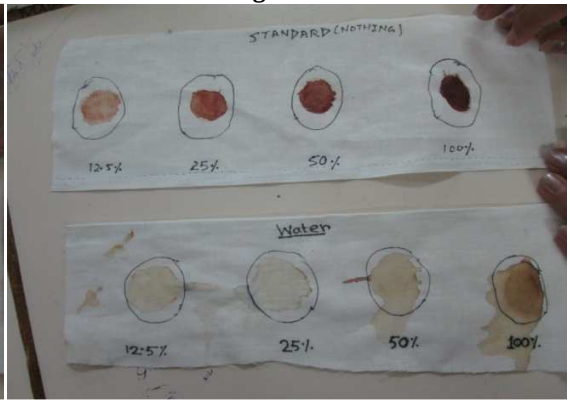


Fig.5:



CONCLUSION

Dairy sludge is one of the potential sites which may contain bacteria. The sludge environment provides a inoculum's concentration and it is good source for proteolytic microorganisms to flourish. In this study the dairy sludge samples were screened for accordance with Elibol and Moreira et al.[16] bacteria producing alkaline proteases by the screening. The formation of clear zones around the colonies confirmed the production of alkaline protease. five isolates showed a clear zone in skim milk agar plate Although many potent strains are on market for enzyme production but we preferred this isolate because they could be alternative for commercial use. The results of the present study evidenced that the production of value-added products like proteolytic enzymes using dairy sludge is possible. By reusing such industry sludge and effluents as substrate for enzyme production will helps in the reduction of production cost and also helps in enhanced production of valuable bioproducts.

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