JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue
JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)
An International Scholarly Open Access, Peer-reviewed, Refereed Journal

The production, optimization and partial purification of protease enzyme from *Bacillus aryabhattai* KJAM03

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Abstract: The increasing interest in green synthesis of products has led to an increased demand for microbial enzymes in industrial processes. In the present study, a protease-producing strain was isolated from a contaminated soil sample. The protease producers were screened on skim milk agar plates and the protease activity was confirmed using the Folin-Lowry method. Among the four isolates obtained in our study, *Bacillus aryabhattai* KJAM03 (NCBI accession no. LC337962) showed maximum protease production (32.12 U/mL). The 'one factor at a time' approach was followed to optimize the enzyme production from *B. aryabhattai* KJAM03 and a 73.17% improvement was achieved. The optimum conditions for enzyme production were identified as 1mL inoculum, 30°C, shaker conditions (130rpm) and 72h incubation in G medium (pH 7) containing peptone (5% w/v), casein (5% w/v) and 0.51% NaCl. The absence of peptone or casein reduced the protease production by 60.4% and 22.4% respectively. The enzyme activity was found to be stable between pH 6-10 and temperature up to 75°C. Although the loss of enzyme activity occurred in the presence of metal ions and surfactants, the enzyme showed excellent stability and compatibility with common laundry detergents (Ariel, Surf Excel and Tide). It also removed blood stains efficiently from cotton fabrics. Overall, our findings suggest a practical application of protease produced by *B. aryabhattai* KJAM03 in detergent formulations.

Index terms: *Bacillus aryabhattai*, Detergent, Optimization, Protease, Surfactant

I. INTRODUCTION

Proteases (EC 3.4.21–24) are hydrolytic enzymes that can metabolize proteins into simple components. The areas of commercial applications of proteases include industrial (food, leather, dairy, detergent, cosmetics), biotechnological (waste management) as well as medicinal (pharmaceutical and diagnostic) sectors (de Souza *et al*., 2015; Zambare *et al*., 2011). These enzymes conquer over 60% of the industrial enzyme market, globally (Shankar *et al*., 2011). It is also a vital enzyme essential for various metabolic and biochemical processes like energy consumption, cell growth and differentiation. Hence, it is a ubiquitous enzyme in nature produced by all the members of the plant and animal kingdoms. Among microorganisms, although a diverse group is known to produce protease, more valuable sources can be traced to the genera Streptomyces and Bacillus (Asker *et al*., 2013; Chander and Puri, 2019). Bacterial proteases, in general, have emerged as an excellent choice for the food industry due to their efficiency in preventing bitterness. They are mostly extracellular, thermostable, and active at a wide pH range. They also show a high affinity for hydrophobic amino acid pairs and broad substrate specificity. The neutral bacterial proteases are active in a narrow pH range (5-8) and show less tolerance to changes in temperature. These characteristics are further beneficial in controlling their reactivity during the production of food hydrolysates (Chander and Puri, 2019).

Several economically important industrial enzymes are produced by *Bacillus* spp. (Hoa *et al*., 2000). They are known to produce a variety of extracellular metallo- or serine proteases. The former enzymes require divalent metal ions for their activity whereas the latter are not affected by chelating agents. A few examples of reported protease producers include *Bacillus cereus*, *Bacillus sterothermophilus*, *Bacillus mojavensis*, *Bacillus megaterium* and *Bacillus subtilis* (Sookkheo *et al*., 2000; Beg and Gupta, 2003; Banik and Prakash, 2004; Gerze *et al*., 2005). The proteases produced by all these isolates were characterized to be superior in activity. They also showed significant stability, broad substrate specificity and a shorter period of fermentation. A simple and cost-effective downstream purification of enzymes produced by bacterial isolates has also been demonstrated (Haddar *et al*., 2009; Maurer, 2004).

The alkaline proteases, especially the ones showing compatibility with detergents at a high temperature, find extensive application in the detergent industry (Belhoul *et al*., 2015). This requires their activity in the presence of surfactants, bleaching agents and other formulation aids. They facilitate the release of proteinaceous materials from fabric stained with milk, blood, egg, meat, fish and body secretions. They also improve the washing performance of solutions used for cleaning contact lenses or dentures (Grbavčid *et al*., 2011; Baweja *et al*., 2016). Hence, these enzymes have been popular in the detergent industries for over 50 years and account to 25–30% of total industrial enzyme requirement (Singh *et al*., 2016).

The objectives of our study were to screen protease producers from various samples and optimize the enzyme production by the most promising isolate. In addition, we also studied the parameters required for optimum enzyme activity and its application as a detergent additive.

II. MATERIAL AND METHODS

2.1. Sample collection

The samples were collected from various sources like spoiled fruits, vegetables and dairy products, oil-contaminated soil and contaminated honey for screening of protease-producing bacteria. The samples were collected in sterile plastic bags, transported to the laboratory and processed immediately.

2.2. Enrichment, screening and isolation of protease producers

The samples (1g) were suspended in 100mL of sterile Nutrient Broth (NB; pH 7), mixed thoroughly and incubated at 30°C for 24h under shaker (120rpm) conditions. The enriched broth was then used for screening of protease producers on sterile skim milk agar plates. The plates were incubated at 30°C for 24h to observe for isolated colonies showing a zone of clearance. These isolates were further purified and maintained on Nutrient agar slants at 4°C. The protease activity of each isolate was also assayed.

2.3. Protease assay

The proteolytic activity of enzymes produced by different isolates was assayed using the Folin-Lowry method with some modifications (Aruna *et al*., 2014). The isolates screened in our study were inoculated in 50mL of sterile NB and incubated at 30°C for 24h under shaker (120 rpm) conditions. The cells were separated from the broth by centrifugation at 5000 rpm for 20 min and the supernatant containing the crude enzyme extract was used for the assay. The crude enzyme (1mL) was allowed to react with substrate (9 ml) for 20 min. The reaction was terminated by adding 1.5mL (5%) trichloroacetic acid (TCA; stopping reagent). The reaction mixture was then centrifuged at 5000 rpm for 20 min and the supernatant was used to determine the amount of TCA soluble amino acids released. Necessary controls and enzyme blank were also maintained throughout the assay. The absorbance was measured at 670nm (Lowry *et al*., 1951) and used to plot a standard tyrosine graph. One unit of activity was calculated as µg/mL of tyrosine produced per minute per mL of enzyme under standard assay conditions (Ibrahim *et al*., 2015).

2.4. Identification of protease-producing bacteria

The potential isolate showing maximum protease activity was identified preliminarily by morphological, cultural and biochemical tests. The strain was confirmed by 16s rRNA gene sequence analysis. PCR-based 16S rRNA gene amplification and sequencing of the isolated bacterium was carried out using universal primers at Sai Biosystems Private Limited, India.

2.5. Optimization of parameters to enhance protease production

The enzyme production is greatly influenced by physicochemical parameters such as pH, incubation temperature, incubation time, agitation and inoculum size. The optimization of these parameters was done by applying one factor at a time (OFAT) approach. In this method, one variable of the system is changed at a time while keeping the others constant. The initial parameters used in our study were NB medium (50mL), 30°C, shaker conditions (120rpm) and 1mL inoculum adjusted to 0.2 $O.D_{540nm}$. Besides the NB medium, 8 different mediums (A-H) were screened for enhancing protease production. The composition of these media is indicated in Table 2.5. The deletion assay was also done to study the effect of deleted components (one at time) from the production medium. For further experiments, the medium which gave maximum protease activity was used to optimize the physicochemical parameters for the maximum production of protease enzyme. The varying physicochemical parameters optimized in our study included the optical density of test isolate ($O.D_{540nm}$ 0.1 - 0.5), inoculum size (0.1mL and 1–5mL), temperature (30°C-55°C), pH (5-10), incubation period (24-96h) and aeration (0 and 100-160rpm).

Table 2.5. Composition of media used for optimization of protease production

Sr. No.	Media	Composition (g/100mL)	Reference
1	A	Glucose (6), Soyabean meal (2), $CaCl_2$ (0.04), $MgSO_4.7H_2O$ (0.02)	Maghsoodi *et al*., 2013
2	B	Sucrose (1), Soyabean meal (1), Calcium acetate (0.01), KH_2PO_4, (0.1), MgSO4.7H2O (0.01), $CaCl_2.H_2O$ (0.01)	Banerjee *et al*., 1999
3	C	Glucose (0.2), Casein (0.05), Peptone (0.05), Yeast extract (0.05), Na_2CO_3 (1), KH_2PO_4 (0.5), $MgSO_4.7H_2O$ (0.5), $FeSO_4.7H_2O$ (0.01)	Rathod and Pathak, 2014
4	D	Glucose (1), Yeast extract (0.5), Peptone (0.5), KH_2PO_4 (0.1), $MgSO_4.7H_2O$ (0.02), NaCl (5), Na_2CO_3 (1)	Ibrahim *et al*., 2015
5	E	Glucose (1), Casein (0.5), Yeast extract (0.55), KH_2PO_4 (0.2), Na_2CO_3 (1), $MgSO_4.7H_2O$ (0.2)	Das and Prasad, 2010
6	F	Casein (1), Tryptone (1), NaCl (1), Yeast extract (0.5)	Kamran *et al*., 2015
7	G	Peptone (1), Meat extract (0.3), NaCl (0.5)	Suganthi *et al*., 2013
8	H	Glucose (1), Casein (0.5), Yeast extract (0.5), KH_2PO_4 (0.2), K_2HPO_4 (0.2)	Sharma *et al*., 2014

The influence of carbon sources like 1% (w/v) glucose, sucrose, xylose, ribose, arabinose, starch, galactose, lactose, mannitol, maltose, mannose, glycerol and fructose were studied on protease production. Similarly, the effect of organic nitrogen sources (yeast extract, tryptone, beef extract, soybean meal, meat extract and peptone) and casein was also studied by replacing 1% peptone and meat extract in the original production medium. In addition, the effect of increasing concentration of suitable nutrient sources on protease production was observed (Vijayalakshmi *et al*., 2011; Devi *et al*., 2008).

2.6. Purification of the enzyme

The crude enzyme extract was purified by the ammonium sulphate precipitation method. Different concentration of solid ammonium sulphate (40-90%) was added to the crude enzyme and incubated overnight at 4°C. The precipitate was collected by centrifugation at 5000 rpm for 20 min under refrigerated conditions. It was then re-suspended in a 5mL of chilled 50mM potassium phosphate buffer (pH 7) and then subjected to dialysis against the same buffer.

2.7. Study of the effect of various physicochemical parameters on purified enzyme

Protease enzyme from the most promising isolate was assayed to determine the optimum conditions of temperature, pH, metal ions, organic solvents, surfactants and detergents. The protease activity was assayed at various pH (4-10.6) and temperature (30°C-85°C). The effect of various metal ions (5mM) such as Ca^{2+}, Cd^{2+}, Zn^{2+}, Hg^{2+}, Mg^{2+}, K^{+}, Na^{+} and Fe^{2+} was also investigated on protease activity. The metal ions were used in the form of $CaCl_2$, $CdCl_2$, $ZnCl_2$, $HgCl_2$, $MgCl_2$, KCl, NaCl and $FeCl_3$. Similarly, the effect of various organic solvents (1%) such as benzene, ethanol, methanol, iso-2-butanol and DMSO was investigated on protease activity. The detergents such as SDS, Tween 80, Ariel, Wheel and Surf Excel were also used to study their effect on protease activity.

2.8. Application of protease as a detergent additive

The application of protease as a detergent additive was studied using white cotton cloth pieces (3x3cm) stained with human blood. The stained cloth was dried overnight and then treated with various detergent solutions (7mg/mL) of Ariel, Surf Excel and Tide. These were then heated in a boiling water bath for 20 min to inactivate the inherent enzymes (Kanmani *et al*., 2011).

The following sets were prepared to compare the efficacy of the test protease enzyme with commercial detergents.
1) Flask with distilled water (100 mL) + stained cloth.
2) Flask with distilled water (100 mL) + stained cloth + 1 mL of detergents.
3) Flask with distilled water (100 mL) + stained cloth + 1 mL of aerial detergents + 1 mL (1:5 diluted) enzyme solution.

The above flasks were incubated at 55°C for 15 min. After incubation, the cloth pieces were taken out, rinsed with water and dried. The efficacy of protease in stain removal was done based on a visual examination of rinsed cloth samples. Untreated cloth piece stained with blood was used as a control (Banerjee *et al*., 1999; Aruna *et al*., 2014).

2.9. Statistics

All of the experiments were carried out in triplicate, and the mean values with standard deviation were reported.

III. RESULTS

3.1. Screening, identification and growth characteristics of potential protease producer

From among the ten isolates, four isolates showed a zone of clearance on skimmed milk agar plates thus confirming their proteolytic activity. Fig. 3.1 represents the protease activity of all 4 isolates. The isolate 2 exhibited maximum protease activity (65.3U/mL) and hence was selected for further optimization studies. Based on morphological, cultural, and biochemical tests and 16S rRNA sequence analysis, it was identified as *Bacillus aryabhattai* KJAM03 (Accession no. LC337962). The enzyme production by *B. aryabhattai* KJAM03 gradually increased with the passage of time and the highest enzyme activity was obtained at 72 h of incubation, prolonged incubation decreased the enzyme activity; however, the growth of the microorganism was not significantly affected.

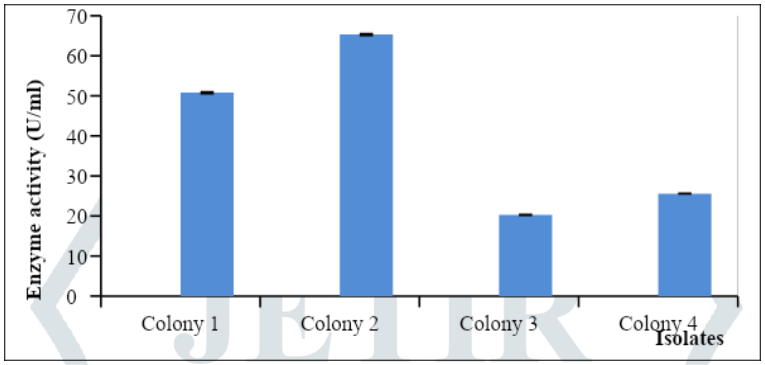

Figure. 3.1. Enzyme activity of protease produced by screened isolates

3.2. Optimization of production media to enhance protease production

The extent of enzyme production supported by different media is represented in Fig. 2. *B. aryabhattai* KJAM03 exhibited maximum production of protease in G medium (32.12 U/mL). In order to evaluate the effect of individual components, a deletion assay was also performed. It indicated that the absence of meat extract and mannose lowered the protease yield by 15.16 and 12.7% respectively. Whereas, deleting peptone and casein from the production media significantly reduced the protease yield by 60.4% and 22.4% respectively (Fig. 3.2.a/3.2.b.). These observations further explain the low yield of protease observed in medium C which lacked peptone, meat extract as well as casein.

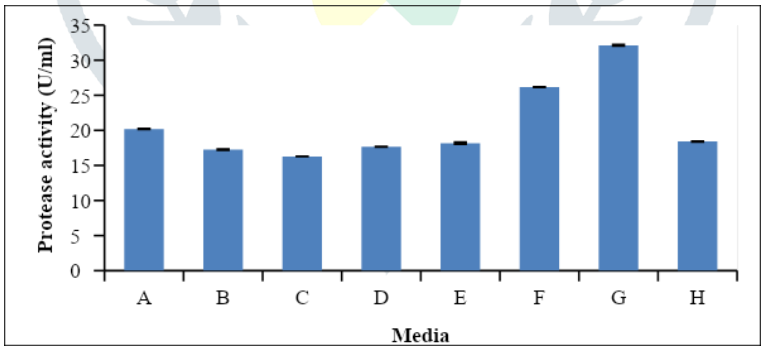

Figure. 3.2.a Optimization of media for protease production by *B. aryabhattai* KJAM03

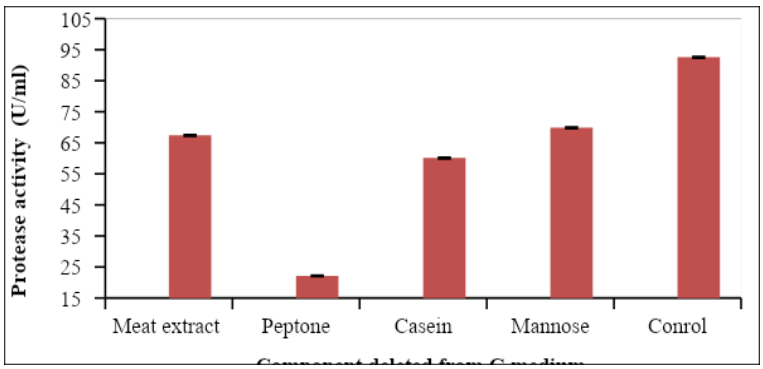

Figure. 3.2.b Effect of deleted components from G medium on protease production by *B. aryabhattai* KJAM03

3.3. Optimization of physicochemical parameters to enhance protease production

The Figure 3.3 represents the physicochemical parameters required for optimum protease production from *B. aryabhattai* KJAM03. These conditions were identified as 1mL inoculum in G medium (pH 7), 30°C, shaker conditions (130rpm) and 72h incubation. A decrease in enzyme production after 72h may be due to its inactivation by metabolic end products accumulated in the medium or nutrient depletion (Shumi *et al*. 2004).

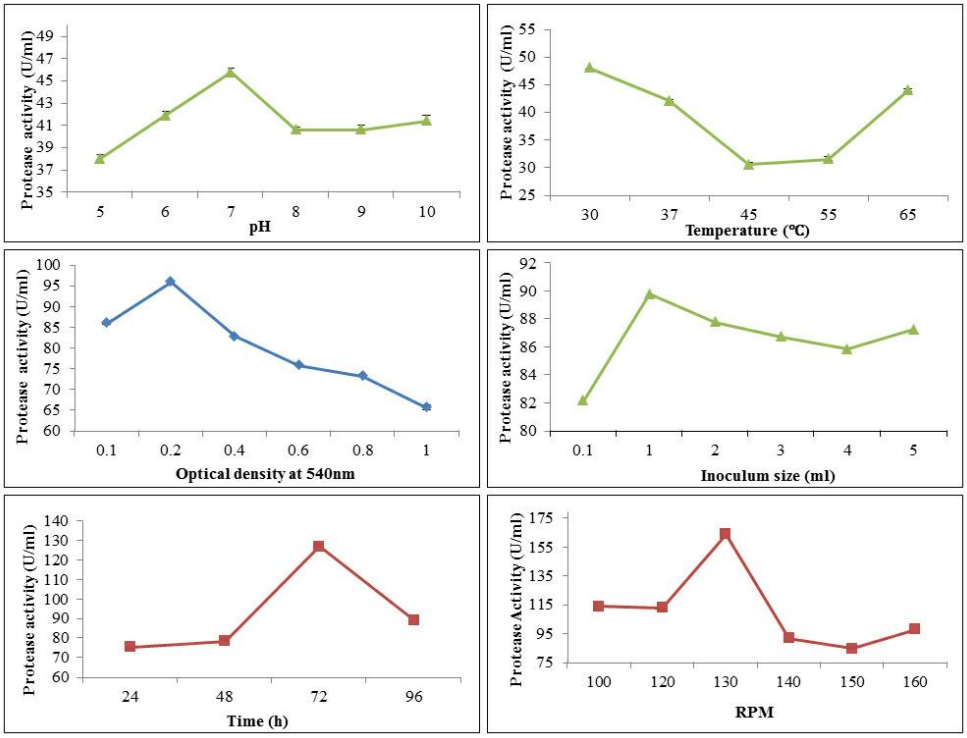

Figure. 3.3. Optimization of physicochemical parameters for protease production by *B. aryabhattai* KJAM03

3.4. Optimization of carbon and nitrogen sources to enhance protease production

The Figure 3.4 represents the effect of various carbon and nitrogen sources on protease production from *B. aryabhattai* KJAM03 and the optimization of nutrient concentration. Although enzyme production was observed in the presence of sugars like mannose, fructose, mannitol, maltose, galactose, starch, lactose, arabinose and ribose, it was significantly lower than that obtained in their absence. On the other hand, 5% peptone improved the yield of protease enzyme by 10%. However, maximum yield was obtained on the addition of 5% casein, as a substrate, to the production medium.

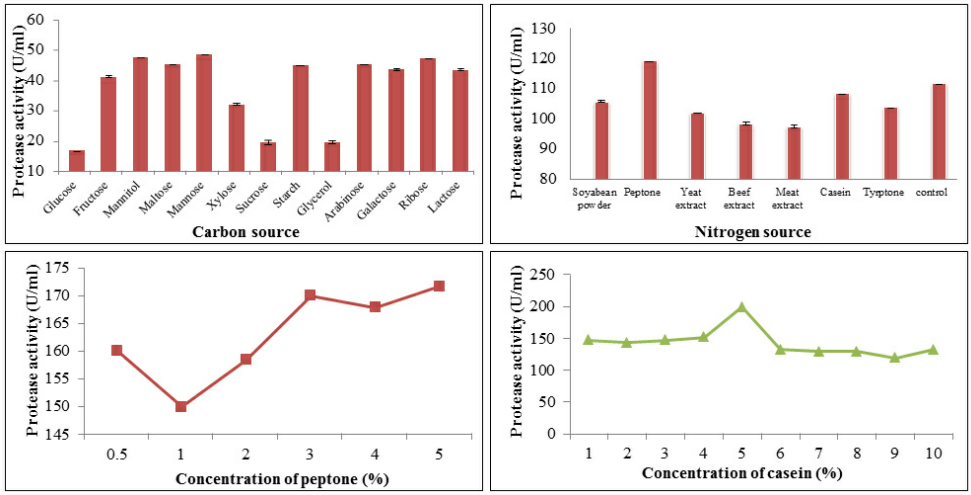

Figure. 3.4. Optimization of carbon and nitrogen sources for protease production by *B. aryabhattai* KJAM03

3.5. Effect of various parameters on the activity of the purified enzyme

The protease enzyme was partially purified by ammonium sulphate precipitation which shows 76 U/mL, whereas after dialysis the protease enzyme purified and showed 88.8 U/mL with a specific activity of 3.7 U/mg. Thus, dialysis not only facilitated the effective

removal of the impurities from the enzyme but also helped in concentrating it. On further analysis, we found that *B. aryabhattai* KJAM03 was active over a broad range of pH (4 to 10) and temperature (between 30ºC- 85ºC) with optimum activity observed at pH 7 and 30ºC (Fig. 3.5). The metal ions are required for optimum function of some enzymes. Hence, the effect of various metal ions with 5mM conc. on protease activity of *B. aryabhattai* KJAM03 was studied. It was observed that the addition of Ca^{+2} and N^{+} ions showed 85.56% and 84.13% residual activity respectively. Whereas, other metal ions like Hg^{+2}, Zn^{+2}, Cd^{+2}, Fe^{2+}, N^{+} and K^{+} showed less residual activity result a reduction in protease yields. Hence, Mg^{2+} showed 71.4% residual activity which reduced protease yield drastically (Fig. 3.5). Similarly, the enzymes are usually inactivated by the addition of organic solvents to the reaction solution. The protease activity of *B. aryabhattai* KJAM03 was reduced with the addition of benzene (97.27 % residual activity). More than 70% of activity was retained with acetone, butanol, methanol, DMSO and ethanol (Fig. 3.5).

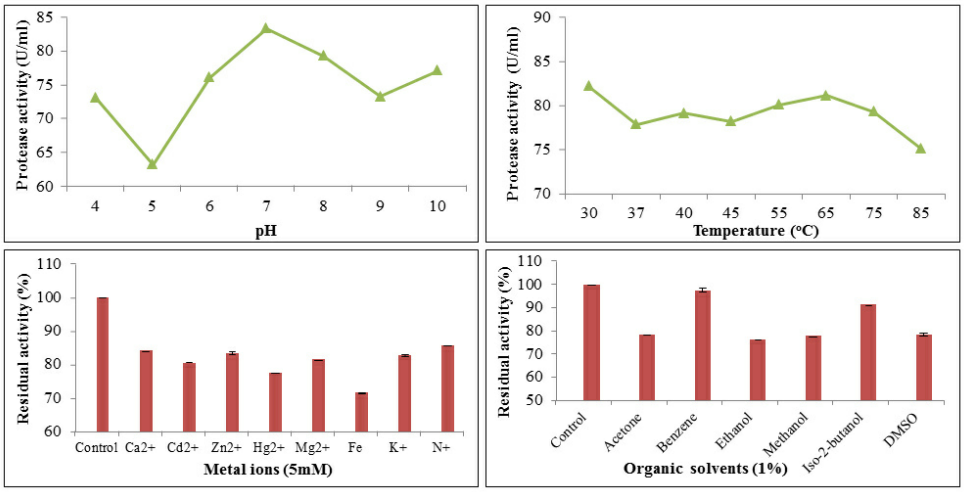

Figure. 3.5. Effect of different parameters, supplements and inhibitors on protease enzyme activity

3.6. Application of protease as a detergent additive

The purified enzyme was tested for its compatibility with commercially available detergents. Since the protease from *B. aryabhattai* KJAM03 showed activity over a broad pH and temperature range and compatibility with various surfactants and solvents, its application as a detergent additive was studied on white cotton cloth pieces stained with human blood. The effect of the organic solvent and different detergents was carried out for the protease enzyme yield (Fig.3.6.a). The activity of protease was retained in the presence oof rganic solvents with (94.67%) & (80.31%) residual activity in Tween-80 & SDS. However, the detergents also showed maximum stability with protease enzyme with residual activity in Tide (90.12%), Surf Excel (73.82%) and Ariel (70.33%). The stability pattern of protease in the presence of commercial detergents (Fig. 3.6.a) also reveals its possible commercial application in detergent formulations. Supplementation of protease with Tide showed better stain removal ability (Fig. 3.6.b.). This provided evidence that the enzyme under consideration has a potential detergent action. Hence, *B. aryabhattai* KJAM03 enzyme preparation could be considered a potential candidate for use as a cleaning additive in detergents to facilitate the release of proteinaceous stains.

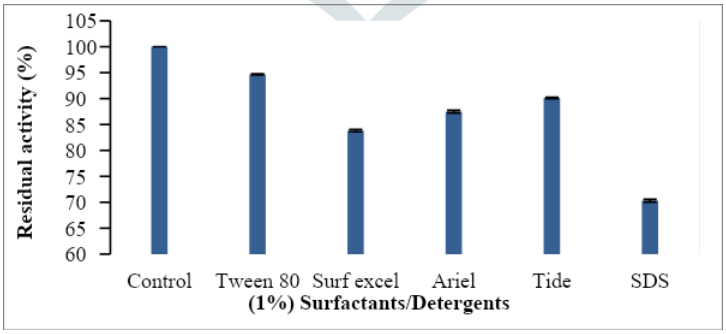

Figure. 3.6. a. Effect of surfactants and detergent on protease enzyme activity

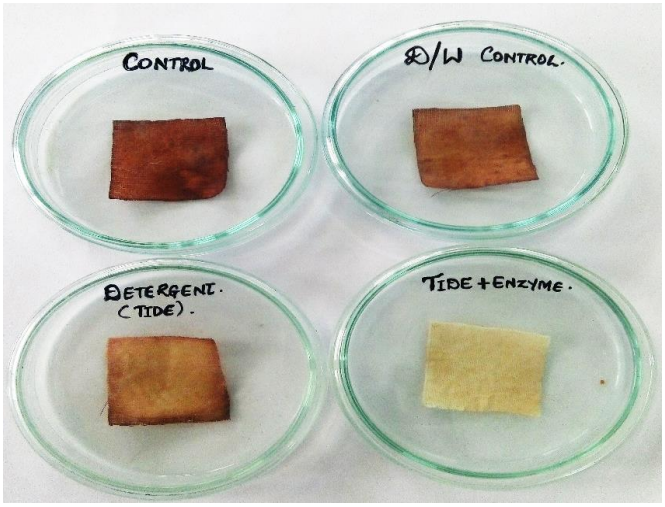

Figure. 3.6.b. Compatibility of protease with Tide and its efficacy in stain removal

IV. DISCUSSION

Many factors like metabolic activity, mechanisms operative during their transition between the exponential and stationary phase and nature of the enzyme (constitutive or partially inducible) affect its production in bacteria. In turn, these factors are influenced by the growth medium. Hence, selection of suitable media is of great significance in the optimization of enzyme production. Several undefined media have been reported in the literature for optimizing protease yield from bacteria. No defined medium has been established so far due to the strain-specific adaptability and nutrient requirements that occur in nature (Sharma *et al*., 2017). Common media like NB (Suganthi *et al*., 2013), Luria bertani broth (Kamran *et al*., 2015) and skim milk agar (Jeyadharshan, 2013) have been reported for the production of proteases from different microbial sources. In addition, mineral medium with casein (Milal *et al*., 2016) and divalent metal ions (Radhakrishnan and Nagarajan, 2012) have shown significant protease production from *Bacillus* spp. In fact, few studies have reported the negative effect of deleting Ca^{+2}, K^{+2} and Mg^{+2} ions from the production media, on protease production (Aruna *et al*., 2014; Manavalan *et al*., 2020; Qadar *et al*., 2009).

The pH and temperature parameters affect the stability of the media components. Specifically, the temperature is critical for optimum metabolism, whereas pH influences many enzymatic processes and the transport of components across the cell membranes. Thus, they support the cell growth and formation of desired products. Optimum inoculum density prevents competition for nutrients, and aeration supports growth by maintaining the desired oxygen level. Similar to our study, *B. polymyxa* and *B. thuringiensis* required pH 7 and 30°C for optimum production of protease (Maal *et al*., 2009; Stefanov *et al*., 2018). Higher temperature and pH negatively affect the metabolic activities of microorganisms, except for extremophiles adapted to grow under these conditions. A few examples of these bacteria include *B. subtilis* K-1 (60ºC; Singha and Bajaj, 2015) and *B. circulans* MTCC 7942 (pH 10; Patil and Chaudhari, 2013). Also, although the agitation of 100-150 rpm has been reported as ideal for bacterial growth and metabolism (Jayaraman *et al*., 2012; Olajuyigbe 2013; Ibrahim *et al*., 2015), *Bacillus* spps. B21-2 showed an exceptional observation of optimum protease production at 600 rpm (Fujiwara and Yamamoto 1987).

Like the physicochemical parameters, the carbon and nitrogen requirement also differs in bacterial spps. For instance, glucose supported protease production by *B. pseudofirmus* AL-89, whereas its addition suppressed protease yield from *Nesterenkonia* sp. AL-20. The catabolic repression mechanism is commonly known to suppress enzyme production in bacterial species, making carbon sources ineffective in increasing the yield. Many authors have also reported repression in protease synthesis on the addition of glucose, maltose or other simple sugars (Zambare *et al*., 2011; Kanekar *et al*., 2002). In contrast, Kumar *et al*. (2014) reported maximum protease production (622.64 U/mL) with lactose (10g/L) in the production medium for *B. aryabhattai* K3. Pant *et al*., (2015) also reported 1% galactose to aid in protease production. Our observations, along with the reported findings, thus validate the significance of optimization studies in evaluating the growth and metabolic requirement, and characterization of novel bacteria.

B. aryabhattai KJAM03 was active over a broad range of pH (4 to 10) and temperature (between 30ºC- 85ºC) with optimum activity observed at pH 7 and 30ºC (Fig. 6). These findings are extremely supportive of the application of

protease as a detergent additive and in tannery industries. For industrial processes, an ideal additive is required to be active in the alkaline pH range and tolerate changes in temperature (Wang *et al*. 2005). Also, the protease which is naturally stable in the presence of organic solvents could be very useful for synthetic reactions.

A good quality protease should also be steady and active in the presence of a range of various surfactants, bleaching agents and detergents (Kamran *et al*. 2015). Hence, the effect of several surfactants and detergents such as SDS, Tween 80, Surf excel, Tide and Ariel on protease activity was studied. Non-ionic detergents have often been proposed to stimulate extracellular protease production by some organisms, due to their potential ability to increase cell wall permeability and release cell-bound enzymes. However, their efficiency is strongly strain-dependent and sometimes they can inhibit protease activity. In the present study protease from *B. aryabhattai* KJAM03 showed good stability with Tween 80 and SDS with 94.67% and 80.13% of residual activity respectively. While other surfactants including commercially available detergents (Surf Excel, Ariel and Tide) exhibited more than 70% residual activity except SDS which showed more than 80% residual activity in (Fig. 7). Our findings of higher enzyme activity with non-ionic surfactants and reduced activity with ionic surfactant are in concordance with other similar studies (Beg *et al*., 2003; Vijayalakshmi *et al*., 2013).

The detergent proteases work best by hydrolyzing large insoluble proteins. Proteins are initially removed from the fabric surface either by components of detergent matrix or by water alone. Depending upon the size of the resulting fragments, they are either solubilized into the bulk solution or deposited themselves back onto the fabric. Hence, the best detergent enzyme provides improved substrate hydrolysis, resulting in better stain removal and anti-redeposition benefits (Hmidet *et al*., 2009). Similar to our study, proteases from other Bacillus spp. have also reported potential detergent action. Few examples include *B. subtilis* (Sharma and Aruna, 2012; Rai and Mukherjee, 2010), *B. cereus* (Kanmani *et al*., 2011), *B. circulans* (Rao *et al*., 2009; Venugopal and Saramma, 2007), *B. pumilus* CBS (Jaouadi *et al*., 2008), *B. brevis* (Banerjee *et al*., 1999), *B. licheniformis* RSP-09-37 (Sareen and Mishra, 2008) and *B. subtilis* (Kumar *et al*., 2012).

V. CONCLUSION

The sustainable approaches are increasingly in demand for industrial processes including the production of valuable enzymes. The optimum conditions of thermostable and pH-stable protease derived from *B. aryabhattai* KJAM03 are presented in this study. It showed excellent compatibility with commercial detergent like Ariel and improved wash performance in stained cloth samples. The ability of the purified enzymes to retain significant activity even in presence of metal ions, detergents and surfactants suggests its eco-friendly application as detergents additives and in tannery industries.

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