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Purification and Characterization of Novel Alkaline Serine Protease from *Bacillus firmus* BAAP-43 isolated from Tannery Industries Soil

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ABSTRACT: **Enzymes are well-known biocatalysts that may economically expedite and facilitate operations in a variety of sectors, including the detergent, food, pharmaceuticals, and biotechnology industries, by catalysing chemical reactions. An extracellular alkaline serine protease from *Bacillus firmus* BAAP-43 was purified by ammonium sulfate precipitation, DEAE- Cellulose and followed by Sephadex G-100. Overall yield was 18.7% and molecular weight of the purified enzyme was 30.2 kDa. pH 9.5, temperature 45°C and 1% NaCl showed maximum enzyme activity. Stability of the enzyme was pH 8.0 to 9.5 and temperature upto 50°C. Enzyme was completely inhibited by PMSF and it is an alkaline serine protease. Ca^{2+} increased the activity of the enzyme and surfactants SDS were slightly inhibited enzyme activity. Among the various organic solvents such as acetone and methanol enhanced the activity. The enzyme showed compatibility with commercial detergents Surf excel improved the cleaning activity. Enzyme showed the high specificity of casein. This study may be the first on the extracellular alkaline serine protease from *Bacillus firmus* BAAP-43. Formulation of detergents is incessantly developed to improve the cleaning effectiveness and to adapt the fulfil market demands. Thus, it can be considered a potentially powerful detergent agent.**

Keywords: Purification, Characterization, Extracellular alkaline serine protease, *Bacillus firmus* BAAP-43.

INTRODUCTION

Proteases are a class of enzymes that catalysis or break down proteins or peptides. As a result of the protease enzymes action on the peptide bonds connecting with the next amino acid residues in a protein molecule, shorter peptides and amino acids were released. (Razzaq *et al*., 2019). Alkaline protease is one of such substitutes with magnified range of applications in detergent, peptide synthesis, food processing, leather, medical diagnosis, pharmaceutical, meat tenderization, silk degumming, baking, brewing, silver recovery, and waste treatment (Al-Dhabi *et al*., 2020). In response to the rising demand and usage, researchers are investigating a number of approaches to identify, redesign, or artificially produce enzymes with better applicability in industrial processes. Due to their widespread availability and rapid pace of growth, proteases derived from microorganisms now predominate in industrial applications. Alkaline proteases and neutral proteases are used for various commercial purposes. The members of the genus *Bacillus*, is the majority of the extracellular alkaline protease producers, from which have been in use for a long time and are often serine proteases (Pathak and Rathod 2018).

Serine proteases and metallo-proteases are two different categories of protease enzyme based on the chemical nature of their active site. Serine proteases are composed of serine residues that form a catalytic triad with an aspartic acid and a histidine at the active site. Serine-alkaline proteases are often not denatured by detergents or hazardous metals and can withstand a broad range of temperature and pH variations (Sundus *et al*., 2016). Serine proteases from *Bacillus* sp. Are widely used in detergent formulations due to their ease of manufacturing, down streaming are gaining prominence in detergent industry (Tekin *et al*., 2020). Microbial sources for enzyme production are preferred for industrial enzyme production because microorganisms are readily available, grow very rapidly, and can be genetically engineered to produce enzymes that perform optimally under a variety of industrial production conditions. Industrial application of alkaline protease demands its considerable activity and stability under diverse antagonistic conditions of extreme pH, temperature, presence of inhibitors, surfactants, oxidizers, and bleaching agents (Chaudhary *et al*., 2021). Aim of this present study was attempts to purification, characterization, and application of alkaline protease from *Bacillus firmus* BAAP-43.

MATERIAL AND METHODS

Assay for proteolytic activity. The activity of protease (caseinolytic) was assayed by a modification method of Kunitz (1947).

Protein determination. Protein concentration was measured by the method of Bradford (1976) using

Bovine Serum Albumin BSA as the standard. During chromatographic purification, protein concentration was estimated by observing the absorbance at 280 nm.

Effects of pH, temperature and NaCl on the activity and stability of purified alkaline protease. In the current study, the activity of the isolated enzyme was measured after the assay mixture was incubated in different pH environments ranging from 3.5 to 11.0 using various buffers at a concentration of 30 mM (Tris-Hcl). The assay procedure was previously described earlier, it was 50 µg aliquot of the enzyme was incubated with the substrate in the required buffer at 45°C for 1 hour. The purified protease was pre-incubated in the aforementioned buffers for 10 hours at the prescribed pH in order to evaluate the pH stability, and residual activity using the previously described standard assay procedure. The reaction mixture comprising 50 µg of the purified enzyme was used to investigate the different temperature for the activity of the enzyme. The residual activity at each temperature was determined by using the standard assay method previously for determine the thermal stability of the enzyme. The purified enzyme was incubated at 40, 50, 60, and 70ºC for various time intervals. The purified enzyme was incubated with substrate in 30 mM Tris-HCl (pH 9.0) with various concentrations of NaCl ranging from 0 to 10% at 45ºC for 1 hour in order to study the effect of NaCl on the enzyme activity. The enzyme activity was assayed after 1 hour of incubation.

Effect of metal ions on the activity of the purified enzyme. Purified alkaline protease was incubated for 1 hour at 45ºC with 30 mM Tris-HCl (pH 9.0) containing various metal ions, including Mn^{2+}, Mg^{2+}, Ba^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Na^{+}. K^{+} and Zn^{2+} at a final concentration of 2 mM. The activity which doesn't contain any metal ions was regarded as the control (100%) activity.

Impact of Organic Solvents on the stability of the purified enzyme. Different organic solvents such as Benzene, Butanol, Methanol, Ethanol, DMSO, Acetone, Hexane, Toulene, Xylene, Isoipropanol, and Choroform at a concentration of 25% were added to an aliquot (50 µg) of purified protease in 2 ml of Tris-HCl buffer (30 mM; pH 9.0). The reaction mixtures were incubated at 45°C for 1 hour, after which the residual activity was measured using the standard assay procedure.

Effect of surfactants and enzyme inhibitors on Purified Enzyme. To investigate the effect of surfactants and enzyme inhibitors on enzyme activity, surfactants SDS (0.5%), Triton X-100, Tween 20, Tween 80 and CTAB were added at a final concentration of 5 mM and enzyme inhibitors β-mercaptoethanol and dithiothretiol, as Phenyl methyl sulfonyl fluoride (PMSF), Ethylenediaminetetraacetic acid (EDTA), Idoacetamide, at a concentration of 5 mM, was added to the combination of enzyme and substrate in Tris-HCl buffer (30 mM; pH 9.0), and the mixture was then incubated at 45°C for 1 hour. The residual activity was measured by standard assay procedure.

Influence of detergent stabilityon Purified Enzyme. Detergents such as Wheel, Rin, Ariel, NirmaHenko, Surf Excel, Tide, and Ponvandu at a concentration of (10 mg/ml) were used to evaluate the impact of detergent stability on purified protease in the presence of 30 mM (pH 9.0). Every 30 minutes, the residual activity of the enzyme was measured upto 3 hours at 45°C. Without detergent used as control sample for comparing enzyme activity (Ire *et al*., 2011).

Substrate Specificity of the Enzyme. Casein, gelatin, skim milk, and BSA served as alternative substrates to evaluate the preference of the purified enzyme. The activity against these substrates was measured by standard assay procedure.

RESULT AND DISCUSSION

Enzyme purification and molecular weight determination. Extracellular alkaline protease produced by the *B. firmus* BAAP-43 was purified by ammonium sulphate precipitation, DEAE Cellulose chromatography and Sephadex G-100 gel filtration chromatography. The active fractions collected after gel filtration were analysed using SDS-PAGE to determine their purity. On SDS-PAGE, the purified fraction exhibited a homogenised band. Using known molecular weight markers, the molecular weight found to be 30.2 kDa. Based on the aforementioned purification procedures, the enzyme had a final yield of 18.7%, had been purified 43.1 fold, and had a specific activity of 18360.6 U/mg protein. This study was strongly supported by 30 kDaserine protease from *Mucor subtilissimus* URM 4133 (Gomes *et al*., 2020), 33 kDa protease from *Bacillus alveayuensis* CAS 5 using DEAE-cellulose and Sephadex G-50 columns (Annamalai *et al*. 2013), whereas Jellouli *et al*. (2011) reported 30 kDa protease from *B. licheniformis* MP1 using Sephadex G-100 and Mono Q-Sepharose columns.

Influence of pH on the activity and stability of the purified alkaline protease. The highest activity of purified protease was observed at pH 9.5, while it was active over a wide pH range of 4.5 to 11.0. Maximum activity of 18379.4 U/mg protein was recorded at pH 8.5 to 9.5 and 18011.8 U/mg protein was recorded at pH 10.0. The enzyme activity gradually decreased slightly above pH 10.0 and below pH 8.0 (Fig. 1). The stability of the purified alkaline protease was tested at various pH levels; after 10-hour incubation, the highest residual activity of 97% was found at pH 9.5, which was followed by pH 8.0 to 9.0. During 10-hour incubation, over 50% of the residual activity was found at pH 11.0, whereas over 27% of the remaining protease activity was found at pH 4.5 (Fig. 2). Similar results were reported by Zaman *et al*. (2023). These findings are in accordance with the alkaline proteases, the optimum activity and stability of the protease which was at the range of 7.0–10.0 in the previously reported (Vildan *et al*., 2017). Similarly, optimum pH as 9.0 for three alkaline proteases from *B. licheniformis* reported by Manachini and Fortina (1998) was lower than that of BA17 alkaline protease. Sellami-Kamoun *et al*. (2008)

reported the similar type of alkaline protease from *B. licheniformis* RP1.

Effect of temperature on the activity and thermal stability of the purified protease. The maximum activity of purified alkaline protease was 18385.5 U/mg protein was obtained at 45°C, which was followed by 50°C and 40°C. At 4°C, the protease activity was absolutely lost, and at 70°C, it was inhibited more than 50%. (Fig. 3). The purified protease's thermal stability was shown to be unchanged after 3 hours of incubation at 40°C to 50°C. Activity of enzyme was slightly reduced by pre-incubation at temperatures greater than 55°C. Incubation at more than 65°C resulted in 50% of loss of activity (Fig. 4). Our study was strongly supported by Lu *et al* 2022 demonstrated that the protease was stable within 100 min at 45–55◦C and maintained 80% enzyme activity. Behzad *et al*. (2014) reported that the extracellular protease from *Bacillus* sp was found to be more stable at 40-60ºC, since it retained greater than 75% of the initial activity after 1 h incubation the stability decreased to 65%° at 70 and 80 and 53%, respectively.

Effect of sodium chloride concentration on the activity of purified protease. The purified enzyme had maximal activity at 1% NaCl concentration and maintained more than 50% of activity at 2% to 5%, while activity dropped by 25% at 10%. Enzyme action was lost at increasing concentration NaCl (Fig. 5). The addition of 5% salt had no effect on the enzyme's activity. Yet, when the quantity of NaCl increased, its activity rapidly reduced (Neyssene *et al.*, 2017). The addition of 5% salt had no effect on the enzyme's activity. Yet, when the quantity of NaCl increased, its activity rapidly reduced. Neyssene *et al.* (2017) The denaturation of the enzyme may provide an explanation for the decline in activity. High salt concentrations actually affect the electrostatic connections between charged amino acids, strengthening the protein-protein interaction that is most likely related to enzyme denaturation and precipitation (Bougatef *et al.*, 2010).

Effect of metal ions on the activity of purified enzyme. Several metal ions were tested in the current study, it was found that Ca^{2+} ions improved residual activity, which was recorded as 148 and 138% in 5 and 10 mM concentration and followed by Mn^{2+}, K^{+}, Na^{+}, Mg^{2+}, Cu^{2+}, Fe^{2+} and Ba^{2+} at the concentration of 5mM and 10mM concentration. The activity of purified protease was slightly inhibited by both 5mM and 10mM concentration of Zn^{2+}, Co^{2+} and Hg^{2+} (Fig. 6). Previously, it has been reported that alkaliphilic *Idiomarina* sp. C9-1 alkaline protease improved enzymatic activity with calcium ions (Zhou *et al*., 2018).

Similarly, serine protease from *Bacillus subtilis* DR8806 was showed K^{+}, Ca^{2+}, Mg^{2+} and Fe^{2+} at 10 mM concentration Whereas, sodium ions showed no considerable effect on enzyme activity (Farhadian *et al*., 2015).

Effect of organic solvents on the activity of purified protease. Various organic solvents has been evaluated at 25% concentration, it revealed that acetone, Methanol, Xylene, Toluene and Isoproponal enhanced the protease activity that 143%, 120%, 110%, 104% and 102% respectively. Whereas Butanol, DMSO, Hexane are slightly inhibited and benzene was more than 50% of enzyme activity inhibited (Fig. 7). A similar kind of stimulatory effect was reported by Ramalingam *et al.* (2022). In contrast Shah *et al*. (2010) reported decane, hexadecane, hexane, cyclooctane, toluene, and benzene could improved the activity of purified protease whereas ethanol and DMSO decreased the activity upto 70% and 80%, respectively.

Effect of surfactants on the activity of purified protease. Effect of surfactants Tween-20 and Triton-X 100 slightly increased the protease activity whereas SDS and CTAB partially (10%) of the protease activity was reduced and Tween 20 inhibited 60% of the protease activity (Fig. 8). Earlier studies demonstrated that non-ionic surfactants, such as Triton X-100 or Tween-20, might either significantly boost or decrease the protease activity when used at increasing levels (Yang *et al.*, 2020).

Effect of protein inhibitors on the activity of purified protease. Effect of different protein inhibitors such as PMSF, EDTA, β- mercaptoethanol, Iodoacetamide and idoacetic acid and dithiothretiol were studied to identify the chemical nature of enzyme. Among the inhibitors PMSF only completely inhibited enzyme activity whereas EDTA (48%), β- mercaptoethanol (55%), Iodoacetamide (8%) and (23%) at the concentrations of 5 mM were slightly enhanced the enzyme activity (Fig. 9). Tarek *et al.* (2023) reported the serine protease inhibitor PMSF fully inhibited the majority of the alkaline proteases of *Bacillus* species. Most of the Protease enzyme from soil *Bacillus* sp. showed higher activity in the presence of EDTA and iodoaecetae, β-mercaptoethanol and DTT (Moradian *et al*., 2009).

Compatibility of purified protease with detergents. At a concentration of 10 mg/ml, commercial detergents were incubated with the purified protease enzyme for 3 hours at 37°C to assess the impact of detergent stability and compatibility. After incubation in the presence of the surf excel, the enzyme exhibited more than 100% of its activity after 60 minutes of incubation (Fig. 11). In the present study strongly supported the alkaline protease from *Pseudomonas aeruginosa* YPVC was quite stable with detergents at higher temperature (50°C) for longer time period. This property of enzyme provides its suitability for the preparation of eco-friendly cleaning and washing formulations (Chaudhary *et al.*, 2021). After 3 hours of incubation with detergents, more than 60% of the proteolytic activity was present in the presence of the same detergents. Studies on the stability of proteases have already been published in a number of articles, with the stability and activity of the proteases altering in the presence of a variety of commercial detergents (Kuddus and Ramteke 2011). Stability and activity of enzyme in the presence of detergents show the enzyme as potential candidate for utilization in detergent industry as a detergent additive for efficacious stain removal. In the presence of Ca^{2+} and glycine, the protease from *Bacillus brevis*

demonstrated compatibility with commercial detergents such as Ariel, Surf Excels, Surf Ultra, and Rin at 60ºC. Even after three hours at 60ºC, this enzyme exhibited more than 50% of its activity with the majority of the detergents tested (Banerjee *et al*., 1999).

Substrate specificity of the purified protease enzyme. To determine the specificity of the purified protease enzyme, casein, gelatine, skim milk, and bovine serum albumin were tested against the purified enzyme (Fig. 10). This study showed that this protease enzyme was more active towards casein (100%) and followed by skim milk (53%), gelatin (43%) and BSA (6%). This indicates that protease can target a wide range of natural protein substrates, which is in accordance with Elgammal *et al*. (2020) who found that casein is the optimum substrate for protease from *Aspergillus ochraceus* BT21 as compared with gelatin and bovine serum albumin. Our results were highly comparable to result, protease of *Halobacillus* sp. (Yang *et al*., 2013) and *Bacillus amyloliquefaciens* (Olajuyigbe and Ogunyewo 2013) had the highest specificity towards casein.

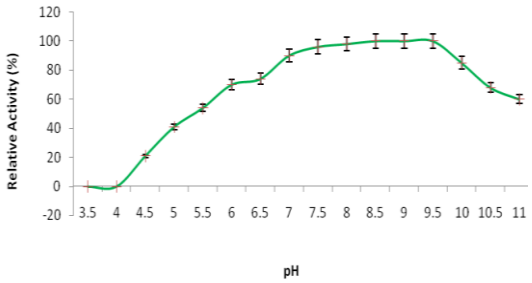

Fig. 1. Influence of pH on the activity of the purified alkaline protease.

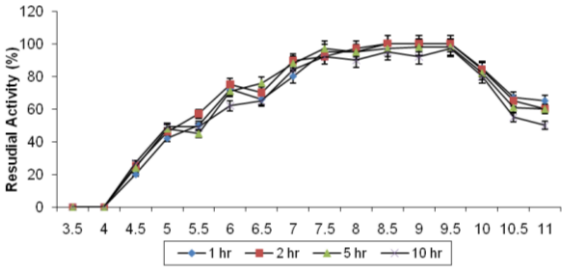

Fig. 2. Influence of pH on the stability of the purified alkaline protease.

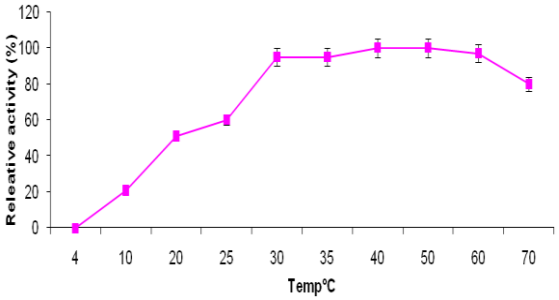

Fig. 3. Effect of temperature on the activity of the purified protease.

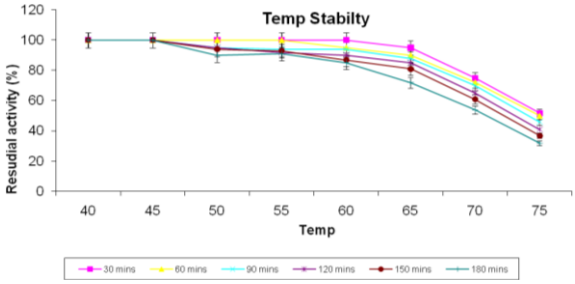

Fig. 4. Effect of temperature on the thermal stability of the purified protease.

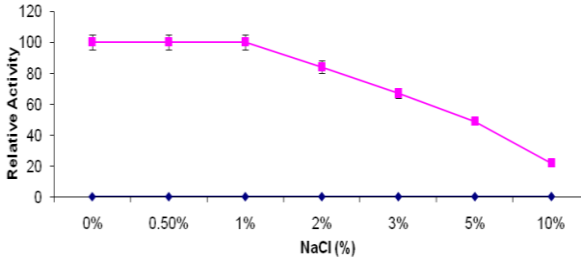

Fig. 5. Effect of NaCl concentration on the activity of purified protease.

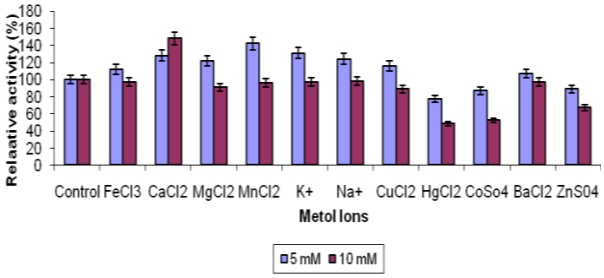

Fig. 6. Effect of metal ions on the activity of purified enzyme.

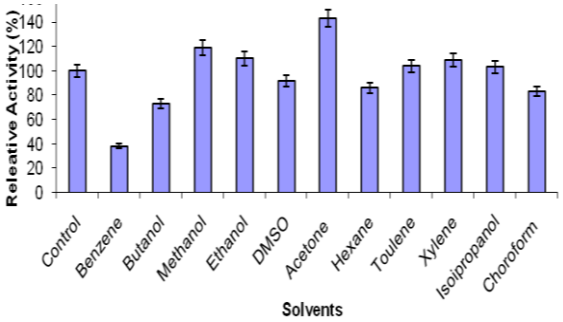

Fig. 7. Effect of organic solvents on the activity of purified protease.

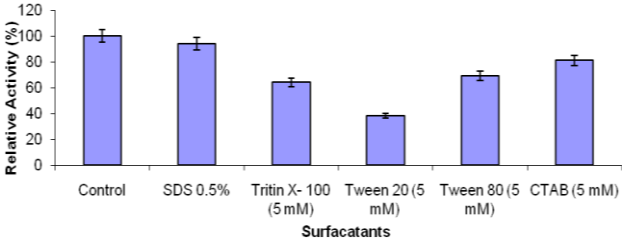

Fig. 8. Effect of surfactants on the activity of purified protease.

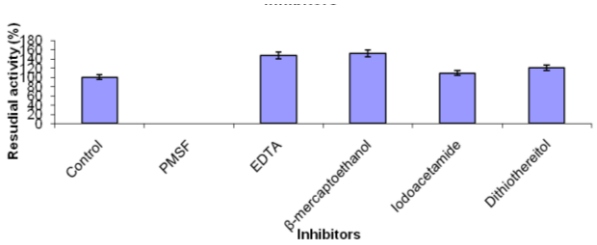

Fig. 9. Effect of protein inhibitors on the activity of purified protease.

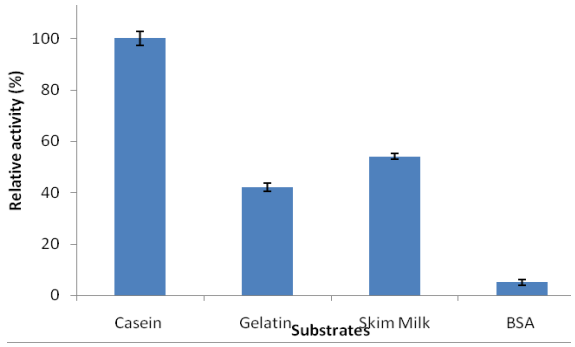

Fig. 10. Substrate specificity of the purified protease enzyme.

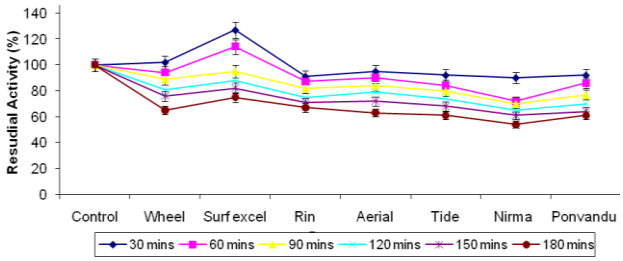

Fig. 11. Compatibility of purified protease with detergents.

CONCLUSIONS

Bacillus species producing alkaline proteases have significant commercial value because of their wide range of biochemical uses in industries like food, medicine, detergents, silver recovery, and waste management. These properties make alkaline proteases as the best and suitable candidate for industrial applications employing higher temperatures along with shorter reaction times and low risk of contamination. The present study was focused on characterization of alkaline serine protease from *Bacillus firmus* BAAP-43. The purified alkaline serine protease has high stability with wide range of pH, temperature, NaCl, metal ions, organic solvents, surfactants, protein inhibitors and detergents. The present investigation showed that the enzyme produced by *Bacillus firmus* BAAP-43 is suitable for commercial exploitation as a detergent agent in detergent industries. It could be further explored for other applications also in many industries

FUTURE SCOPE

Proteases have promising future in various industrial applications is clear. The demand for products made by the detergent business is anticipated to rise even further as the world population continues to rise. Currently, the enzyme production cost is high, which is driving the development towards value-added markets. The microbial enzymes application has allowed the replacement of chemicals used in industries or at home, which prevented the release of toxic substances in the environment. The demand for enzymes with applications in the detergent sector is thus expected to increase, as will the size of the worldwide market for detergent enzymes. Hence, research aimed at developing novel enzymes with industrial uses has to be accelerated in order to meet the need for industrial enzymes. Hence, to address the demand for industrial enzymes, research focused on the creation of novel enzymes with applications in industries needs to be intensified. An enzyme can be employed in a variety of industrial production processes; saving money, time, and resources that would be needed to produce enzymes with various functions.

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