KINETIC CHARACTERIZATION AND INDUSTRIAL APPLICABILITY OF NOVEL PROTEASE PRODUCED FROM *Aspergillus ornatus* USING AGRO-INDUSTRIAL MATERIALS

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In this study, we used various lignocellulosic materials to find out their elicitation potential for induced protease production under solid state fermentation (SSF). A significant difference was recorded in the specific substrate uptake rate, biomass and protease excretion during the exponential phase of *A. ornatus* in the culture medium supplemented with mango peel as a growth supporting substrate. Maximum protease activity of 150 U/mL was recorded after 48 h of controlled fermentation. A purification fold of 11.3 with 269.1 U/mg specific activity and 20.7% yield was achieved after purification in comparison with the crude protease. The characterization profile revealed that purified protease was optimally active at pH 10 and 40 °C. Moreover, the activity of the protease was stimulated up to variable extents by Mn^{2+}, Cd^{2+}, Mg^{2+}, whereas an inhibitory effect was shown by Ethylenediaminetetraacetic acid (EDTA) and Sodium dodecyl sulfate (SDS). A considerable level of compatibility was also recorded against various tested detergents, in addition with up to 48 days of shelf life at room temperature. In conclusion, the maximal production of protease in the presence of a cheaper substrate at low concentration and its potential as a detergent additive for improved washing makes the strain and its enzymes potentially useful for industrial purposes, especially for the detergent and laundry industry.

Keywords: *Aspergillus ornatus*, protease, purification, characterization, detergent compatibility

INTRODUCTION

From the industrial point of view and products with natural origin, proteases (EC 3.4.21-24) are among the largest groups of industrial enzymes. Proteases are mainly classified by the optimal pH in which they thrive; thus there are acidic, neutral, and alkaline proteases.[1-3] Owing to their extraordinary features, proteases are being widely used in several industries, including detergent, diary, leather processing, meat processing, fertilizer, silk industry and many others.[1,4-9] Among the aforementioned protease types, alkaline proteases are robust in nature and have considerable potential to contribute to the development of value-added natural products.[10,11]

Microbial strains, including *Rhizopus*, *Aspergillus*, and *Conidiobolus* spp.,[2,11-15] have the ability to produce proteases under suitable growth conditions.[16] The vast diversity of proteases, in contrast to their mode of action and specificity, clearly illustrates the influence of these enzymes in the biosphere.[17] It is generally considered that most of the microbial proteases are degradative enzymes, which have a noteworthy potential to catalyze the total hydrolysis of proteins.[18] Mostly, proteases have a molecular weight in the range from 40 to 130 kDa.[19,20] These enzymes are found in a wide diversity of sources, such as plants, animals and microorganisms. The inability of the plant and animal proteases to meet the current demands of the modern biotechnological world has led to an increased interest in microbial proteases.[21] Proteases from microbial sources

present almost all characteristics desired for their biotechnological applications.[22] Microbial proteases are predominantly extracellular and can be secreted in the fermentation medium. However, at present, the overall cost of enzyme production is very high (due to the high cost of substrates and media used) and therefore, the development of novel processes to increase the yield of proteases with respect to their industrial requirements coupled with lowering down the production cost is highly appreciable.

Since the industrial use of proteases, particularly the alkaline proteases, are expected to boom enormously in the coming decade, there is a dire demand to search new resources that are cheaper and more cost-effective to enhance the production value and to decrease the market price of this enzyme.[23] In recent years, many researchers around the world are redirecting their research interest towards either cost-free or low-cost feedstocks and/or agricultural wastes as potential substrate(s) for protease production.[1-3,17]

The aim of the present study was to evaluate the feasibility of various substrates in SSF for the production of protease by *A. ornatus*. It was also considered significant to purify and characterize this enzyme to explore the factors affecting culture conditions, productivity, and the properties of protease, thus, we aimed to purify and characterize the protease from *A. ornatus* to present its potential and possible applications for industrial purposes.

EXPERIMENTAL

Chemicals and lignocellulosic substrate

All the chemicals used in this study were of analytical grade unless otherwise specified. Various lignocellulosic substrates, such as corn stover, mango peel, orange peel, banana waste, and apple pomace, were obtained from the local fruit market area of Gujrat, Pakistan.

Pretreatment of substrates

Moisture-free powdered substrates (20 g) were pretreated with 1% HCl in an Erlenmeyer flask (500 mL) at room temperature (30 °C) for 1 h. Afterwards, the substrate slurry was filtered through four layers of muslin cloth, and both filtrate and residue were retained. Residues were washed several times with distilled water to remove extra acidity prior to being used for protease production and further analysis.

Microbial cultures and inoculum development

A. ornatus was available from the Department of Biochemistry and Molecular Biology section of the Institute of Life Sciences, University of Gujrat (UoG), Pakistan, and grown on Vogel's agar slants at 35 °C for 3 days. A pure colony of *A. ornatus* was transferred into 100 mL of Vogel's liquid medium supplemented with trace elements after sterilizing the liquid medium at 103 kPa and 121 °C. The g/L ingredients of the trace element solution were: $ZnSO_4.7H_2O$, 1.0; $CuSO_4.5H_2O$, 0.50; $MnSO_4.H_2O$, 0.50 and $Na_2MoO_4.2H_2O$, 0.50. The inoculated culture was then incubated in a temperature-controlled shaking incubator at 30 ^oC for 20 h at 140 rpm for the development of a homogenous inoculum suspension (1×10^7 to 1×10^8 spores/mL).

Fermentation protocol and protease extraction

During the initial substrate screening trial, 20 g of each substrate was placed in a 500 mL Erlenmeyer flask, 50% moistened with Vogel's media, and inoculated with the freshly developed fungal spore suspension of *A. ornatus*. The inoculated flasks were incubated at 30±1 °C for 5 days. After every 24 h, 100 mL of distilled water was added to the fermented cultures and the flasks were then incubated at 30 °C for half an hour under stirring at 180 rpm. The homogenized media were then centrifuged at 10,000 × g for 10 min and the resultant supernatant was used for analytical studies.

Determination of biomass

The pellets obtained during the extraction process were re-suspended in 50 mM phosphate buffer at pH 7.0 and re-centrifuged at 10,000 × g for 10 min in preweighed falcon tubes and dried at 80 °C until reaching constant weight and final biomass weights in grams were recorded.

Determination of protease activity and protein contents

To determine the protease activity, the method of McDonald and Chen[24] was adopted as reported. Accordingly, a unit enzyme activity was defined as the amount of enzyme that releases 1 µg of tyrosine per mL per min. The amount of protein in the enzyme extract before and after purification was measured according to the method of Bradford,[25] with bovine serum albumin as standard.

Purification of protease

Crude extract obtained from 48 h fermented culture of *A. ornatus* was purified to homogeneity using ammonium sulfate precipitation and fast protein liquid chromatography (FPLC). Crude enzyme was placed in an ice bath after centrifugation at 10,000 × *g* for 15 min at 4 °C to achieve a maximum level of clarity. Solid ammonium sulfate was added to attain 70% saturation to precipitate the protease with continuous stirring for 5 min. The mixture was then kept for 24 h at 4 °C and centrifuged as done previously at 10,000 × *g* for 15 min at 4 °C. The lyophilized pellets were dissolved in 50 mM phosphate buffer (pH 7.0) and

dialyzed against the buffer with constant stirring for 24 h. The dialyzed sample was filtered through a 0.5-µm Millipore filter and loaded onto an anion exchange column RESOURCE-S equilibrated with 50 mM phosphate buffer with a flow rate of 1.0 mL/min. Both the enzyme activity and the protein contents were determined for each fraction, as described in the previous section.

Characterization of protease

Standard quartz cuvettes with exterior width, length and height (W×L×H) dimensions of 12.5×3.5×45 mm were used to determine the values of characterization parameters. The effect of varying pH ranging from 4 to 12 on the activities of protease was investigated. The crude and purified forms were incubated at room temperature (25 °C) for 15 min. The effect of varying temperatures on the activities of protease was also studied. Protease was incubated for 15 min at varying temperatures (20 to 80 °C) before assaying the enzyme activity profile. The effect of various organic and inorganic ions, such as EDTA, SDS, Cd^{2+}, Hg^{2+}, Mg^{2+}, Cu^{2+}, Zn^{+2}, and Mn^{2+}, on protease activity was studied. The Michalis-Menten kinetic constants K_m and V_{max} were calculated from Lineweaver-Burk plots, using casein with varying concentrations (100-1000 µM) as substrate. The enzyme activities in each case were determined under the standard assay conditions as described earlier.

Industrial applicability

Locally accessible detergent brands, namely: Surf excel, Bonus, Ariel, Rin, and Common Surf (locally made) were used to investigate the compatibility of protease. Casein was used as substrate and prepared in phosphate buffer of pH 7.0. A reaction mixture comprising 2.5 mL of substrate solution, 1.5 mL detergent solution, and 1 mL protease was incubated at 25 °C, followed by enzyme assay. To investigate the effect of storage/shelf life of protease on its activity, the enzyme was stored at room temperature (25 °C) for 60 days. The enzyme activities were determined under the standard assay conditions as described earlier.

Statistical analysis

All the experimental data were evaluated in triplicates. The means and standard errors of means (Mean ± S.E.) were calculated for each treatment and represented as Y-error bars in figures, where applicable.

RESULTS AND DISCUSSION

Protease production and substrate screening

Figure 1 illustrates the results obtained during the substrate screening trial. Evidently, it shows the protease productivity (in terms of activity) and changes in the concentration of the carbon source, and dry cell mass as a function of fermentation time in shake flasks. After 48 h of fermentation period, it was observed that the protease continued to be released by *A. ornatus* into the medium, additionally supplemented with mango peel as a growth supporting substrate, at a significant rate and afterwards a decreasing trend was recorded. The protease activity profile obtained from the medium additionally supplemented with lignocellulosic substrates revealed higher enzyme yields than the media without substrate (control) (as shown in Fig. 1). The observed phenomenon clearly indicates the elicitation role of the tested substrate, in addition to its anchoring advantage in the solid state fermentation process. In comparison with other substrates used, this phenomenon was most evident in the case of the fermentation media supplemented with mango peel. The time taken by the culture organism for enzyme synthesis and/or secretion is dependent on the length of the lag phase and primary metabolism on a particular substrate, which greatly varies with the chemical composition and nature of the growth supporting solid substrates, as well as on the method of pretreatment used. It has also been reported in the literature that low-cost substrates, like wheat bran, banana stalk, rice straw, and molasses are suitably effective for microbial growth and enzyme production processes.[26,27] Previously, Sumantha *et al*.[28] reported maximum protease activity after 72 h of fermentation, using *A. niger* as a fermentative microorganism.

Purification of protease

The biosynthesis of protease was carried out in 500 mL Erlenmeyer flasks with a working volume of 200 mL. The cell free crude extract was first purified to homogeneity by three purification steps. The results obtained after each purification step are summarized in Table 1. Firstly, the crude protease extract was precipitated at 70% saturation using solid ammonium sulfate crystals, which revealed 1.41~fold purification with specific activity of 33.7 U/mg. Secondly, ammonium sulfate precipitated fraction of protease was dialyzed against 50 mM phosphate buffer with constant stirring for 24 h and periodic buffer changes.

An ultimate increase in the purification fold and specific activity was recorded with an overall purification fold, and specific activity increase from 1.41 U/mg to 4.18 U/mg, and 33.7 U/mg to 100 U/mg, respectively (Table 1). Thirdly and finally an active protease fraction obtained from

RESOURCE-Q column was further purified to homogeneity by subjecting to FPLC, using a RESOURSE-S column. By FPLC RESOURSE-S column filtration, the enzyme was further purified up to 11.3~fold with specific activity of 269.1 U/mg.

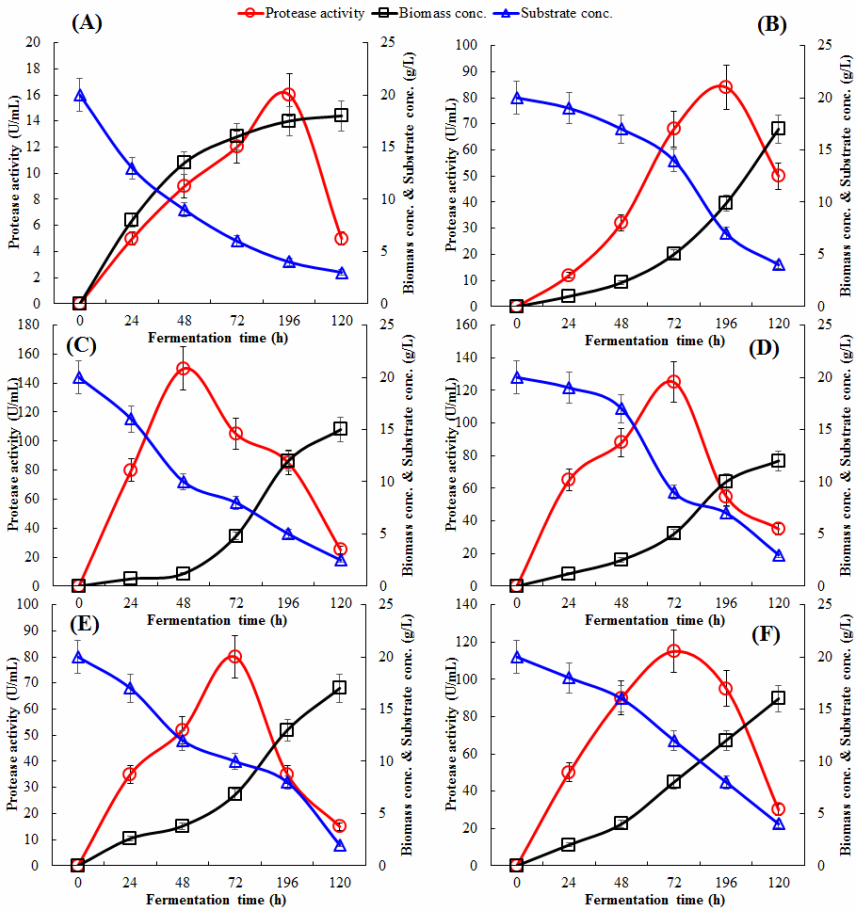

Figure 1: Protease productivity potential of *A. ornatus* using various lignocellulosic based substrates subject to fermentation time period; (A) control without any solid substrate, (B) corn stover, (C) mango peel, (D) orange peel, (E) banana waste, and (F) apple pomace

Table 1
Purification summary of protease produced from *A. ornatus*

Purification steps	Total volume (mL)	Enzyme activity (U)	Protein content (mg)	Specific activity (U/mg)	Purification fold	% Yield
Crude extract	200	43000	1800	23.9	1.00	100
$(NH_4)_2SO_4$ Precipitation	50	15000	445	33.7	1.41	34.9
Ion-exchange chromatography (RESOURCE-Q)	20	12500	125	100	4.18	29.1
FPLC (RESOURCE-S)	10	8880	33	269.1	11.3	20.7

Characterization of purified protease

Effect of pH on protease activity and stability

The pH-activity profile of protease showed that the crude enzyme was optimally active at a pH of 10, whereas in comparison with this, the purified protease displayed noteworthy activity over a broad range from 6 to 10 pH values (Fig. 2 A and B). A consistent decrease in the activity of crude protease was recorded below and above the optimal pH range. The residual activity and stability assay revealed that crude protease was only 19% stable for 24 h, whereas the crude fraction purified up to the level of homogeneity was extraordinarily active and retained its stability up to 72% and 100% for 24 h and 1 h, respectively, which is almost five fold that of the crude fraction (Fig. 2 A and B). The optimum pH of the present protease was different from other fungal species, like *Penicillium camembertii* (pH 3.5),[29] *Rhizopus oryzae* (pH 5.5),[21] and *A. niger* Z1 (pH 9).[30]

Effect of temperature on protease activity and stability

The free form of protease was optimally active at 40 °C, while, interestingly, the purified fraction displayed better activity at a temperature higher than 60 °C (Fig. 3 A and B). A rapid fall in the activity of crude protease was recorded at temperatures higher than the optimal point, when the enzyme started to lose its activity rapidly. The purified protease has an extraordinary thermo-stability for up to 24 h incubation at 70 °C without losing much of its activity, as compared to crude protease (Fig. 3 A and B). Previously, Hussain and co-workers[31] reported maximum protease activity at 40 °C with an overall 80% loss in activity at higher temperature beyond 70 °C. From this perspective, the presently reported protease isolated from *A. ornatus* and purified up to the level of homogeneity is heat-stable and is different from other reported fungal proteases, like that from *A. niger* (32 °C),[32] and *Thaumatococcus daniellii* (35 °C).[33] Relatively higher activity and thermo-stability are among the most attractive and advantageous characteristics of enzymes, which make them suitable for a variety of biotechnological and/or industrial applications.[34]

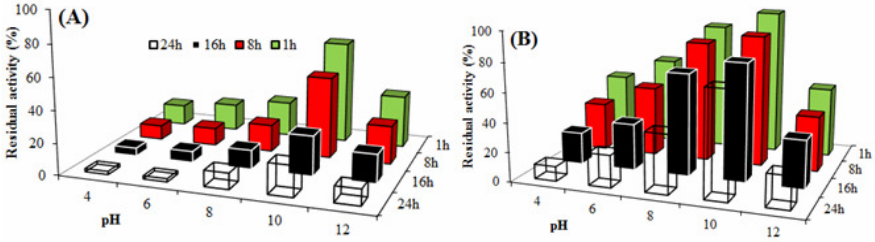

Figure 2: Effect of pH on the activity and stability of (A) crude and (B) purified protease produced from *A. ornatus* using mango peel as growth supporting substrate

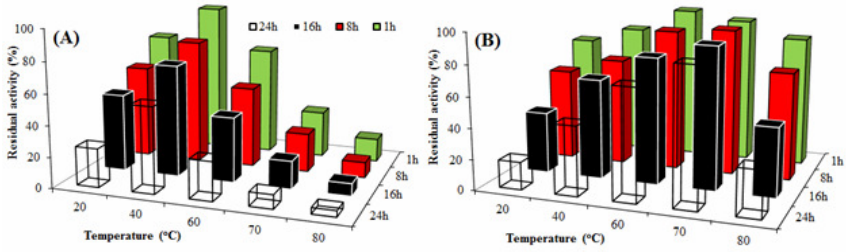

Figure 3: Effect of temperature on the activity and stability of (A) crude and (B) purified protease produced from *A. ornatus* using mango peel as growth supporting substrate

Effect of activators/inhibitors on protease

The results obtained after the standard enzyme assay are shown in Figure 4. It was observed that the crude enzyme only retained 52, and 69% of its activity in the presence of Zn^{2+}, and Mn^{2+}, whereas the purified protease was 100% active in the presence of all those metal activators. A residual activity profile showed that the crude protease was completely inhibited by EDTA, whereas partial inhibition was recorded in the

presence of SDS. In comparison with the crude extract, the purified fraction displayed considerable resistance and thus retained its activity against the aforementioned inhibitory compounds. The various metal ions tested, Mn^{2+}, Cd^{2+}, Mg^{2+}, Cu^{2+}, were observed as being insignificant towards inhibition, as they did not influence the enzyme activity, thus proving that this protease belongs to metalloprotease in nature. This was also confirmed by the inhibition caused by EDTA. Based on the data reported in an earlier study, the addition of Co^{2+}, Mn^{2+}, Fe^{3+}, Ca^{2+}, and Ni^{2+} into the enzyme production medium did not cause any alteration in the enzyme activity.[35]

Effect of substrate concentration: Determination of K_m and V_{max}

When using casein as a substrate, the results obtained were plotted as specific activity (U/mg) against concentration of the casein substrate (µM). The Lineweaver-Burk double reciprocal plot reflects the substrate affinity and catalytic efficiency of the presently reported protease with K_m (48 µM) and V_{max} (133 U/mg) values. As expected, the purification of crude protease up to the level of homogeneity enhanced the specific substrate affinity and catalytic efficiency of the purified fraction of protease, as evidenced by the lower K_m (18 µM) and higher V_{max} (166 U/mg) values, as shown in Figure 5. The lower value of K_m indicates an increase in the substrate affinity potential of the enzyme. There was a slight difference in the K_m and V_{max} values for protease from other reported proteases. The difference in K_m values may be due to genetic variability and substrate specificities among different species.

Industrial application

Prior to recording activities, the purified protease was incubated at 25 °C for 45 min in the presence of each detergent. After the stipulated incubation time (45 min), the enzyme activities were recorded via spectrophotometric analyses and shown in Figure 6. The assay revealed no significant difference in the protease compatibility with Surf Excel and Ariel, however it was observed that the present protease was maximally compatible and retained its activity in the presence of Surf Excel. The observed compatibility phenomenon was followed by Bonus, Rin and then Ariel, whereas the lowest compatibility was recorded in the case of Common Surf (locally made brand). The above discussed results support the compatibility of the present protease with local detergents and suggest that this protease could be a potential candidate for a suitable additive to detergents.

For the possible commercial exploitation of the present protease, the purified enzyme was tested for its storage/shelf life. The effect of storage/shelf life of purified protease on its activity was determined and the results obtained are presented in Figure 7. The enzyme stored at room temperature (25 °C) for up to 60 days revealed that the purified protease was 95% active for 40 days and afterwards a decreasing trend was recorded and the activity was reduced to 62 and 25% after 50 and 60 days, respectively. Evidently, a considerable shelf life and storage values were found, suggesting that this protease may be stored for up to 40 days at room temperature without losing much of protease activity.

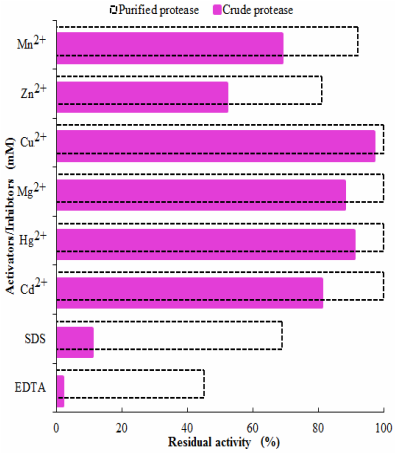

Figure 4: Effect of different activators/inhibitors on crude and purified protease produced by *A. ornatus* using mango peel as growth supporting substrate

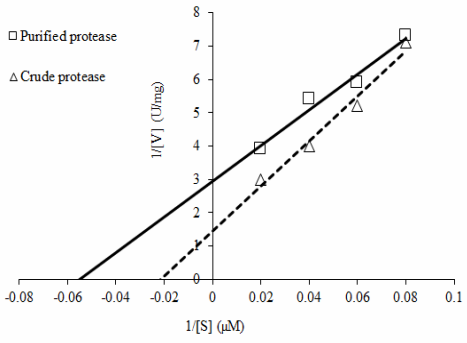

Figure 5: Lineweaver-Burk reciprocal plot: K_m and V_{max} for crude and purified protease produced by *A. ornatus* using mango peel as growth supporting substrate

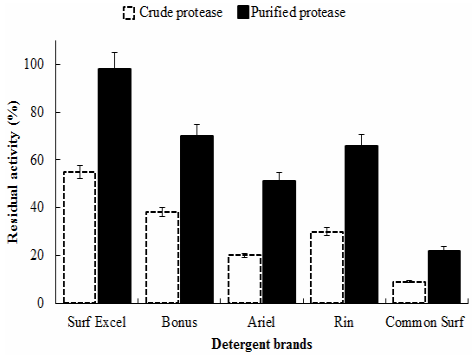

Figure 6: Detergent compatibility of crude and purified protease produced by *A. ornatus* using mango peel as growth supporting substrate

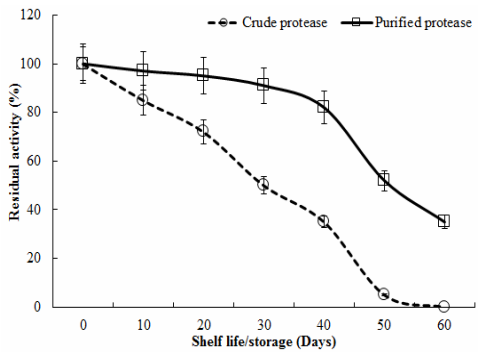

Figure 7: Effect of storage (shelf life) on crude and purified protease produced by *A. ornatus* using mango peel as growth supporting substrate

CONCLUSION

In conclusion, the present approach should be a superior pathway for proper bio-utilization of agro-based waste materials, and their conversion into useful products. *Aspergillus ornatus* used in the present study showed a considerable potential to utilize various substrates as cost-effective microbial growth supporting media. An attempt was made towards finding the best growth conditions for successful cultivation of *A. ornatus* and production of the novel protease enzyme. The characterization results revealed that the present protease was mostly stable over a broad pH and temperature range, which suggests its potential for various biotechnological and/or industrial applications. The purified protease showed a higher level of compatibility with different detergent brands with up to 40 days shelf life at room temperature, suggesting its potential as a valuable detergent additive for improved washing.

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