APPLICATION STUDIES OF PROTEASES FROM COGENT MICROBIAL CONSORTIA OF A COASTAL TROPICAL BIOME FOR QUORUM SENSING INHIBITION AND JUICE CLARIFICATION

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ABSTRACT

Background: Biofilm degradation and fruit juice clarification constitute two highly imperious industrial bioprocesses. Biofilms are the microbial sessile communities embedded in a self-composed polymeric matrix, representing remarkable survival strategy for safeguarding against several dynamic environmental stresses. Quorum sensing inhibition is the subsidence of bacterial cellular communication that impedes their population density and synchrony.Conversely, juice clarification is the breakdown of a semistable emulsion of colloidal plant moieties supporting the insoluble cloudy matter of freshly pressed juice thereby dropping the viscosity and modifying its opacity to an open splotchy look. Enzymatic juice clarification circumvents the comprehensive downstream processing and reduces astringency in taste while improving qualitative characteristics viz. color, viscosity, turbidity and filterability.

Methods: In the present investigation, protease was extracted from persuasive microbial consortia screened from mangrove swamplands and chemically characterized by optimization, stability, spectroscopic, kinetic, inhibition and electrophoreticassays. Application studies of protease were performed to test its quorum sensing inhibition ability and efficaciousness in juice clarification of the indigenous, perishable seasonal fruit Mangifera indica, widely acclaimed for its taste, succulence and exotic flavor.

Findings: The percent biofilm degradation by Crystal violet method was recorded as 90.5% for test sample and 44.5% for positive control. The percent juice clarification, estimated by Bradford method of protein quantitation, was estimated as 18.1% and 60.3% for test sample and positive control respectively.

Coastal tropical biomes are discrete saline shrubland habitats providing idiosyncratic ecostations to diverse microbial communities loaded with hydrolytic enzymes, notably proteases. The escalated cognizance of applications of microbes has solicited the development of newer commercially rewarding biological products in various spheres. Hence microbes from diversified exotic environments have been screened for specific properties expecting to result in novel process applications.

Novelty: The present study is valuable in evolving an additional newfangled approach for the above said pre-eminent commercial bioprocesses.

KEY WORDS: Bioprocess, Ecostation, Mangroves, Biofilm, Bradford, Protease

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1. INTRODUCTION:

1.1Overview

Coastal tropical biomes are discrete saline shrubland habitats providing idiosyncratic eco-stations to diverse microbial communities especially the halophiles.[1]These halophilic organisms are effulgent in a variety of hydrolytic enzymes particularly proteases; with potential applications in industrial bioprocesses.[2] Proteases refer to a bulky cluster of enzymes that conduct proteolysis or the cleavage of proteins; breaking them down into polypeptides or free amino acids by hydrolysis of peptide bonds that connect different amino acids together in the protein chain.

1.2 Quorum Sensing and Quorum Quenching

Communication, as a modus operandi for sharing or interacting between individuals, is considered an innate constituent of organized social behavior.[3] Bacterial communication involves the production, release, detection and response through minute diffusive signal molecules called autoinducers.[4-5] One of these communication phenomena, specific to bacteria, is called Quorum Sensing (QS). Quorum sensing is a cellular communication process that permits the bacteria to share information about cell density and adjust gene expression accordingly[6] thereupon permitting the populations to perform in a synergistic manner and empowering them to conquer their competitors.[7]

The discovery of cell-to-cell communication process between bacteria has steered the understanding that bacteria can also perform co-ordinated activities, which were erstwhile thought to be limited to multicellular organisms. The ability to act collectively as a group has ostensible benefits, such as the capability of migration to a more compatible environment for enhanced nutrient supply and adoption of novel growth patterns, such as biofilm formation or sporulation which can diligently protect them against dangerous environmental impacts. [8]

1.3Biofilms

Biofilms are the microbial clans and communities adhered to biotic or abiotic surfaces and nested in a self-composed hydrated polymeric enclosure. This sessile status represents a remarkable survival strategy for organisms, since it safeguards them against several dynamic environmental stresses like dehydration or starvation and antimicrobial agents viz. antibiotics or biocides.

Although biofilm formation seems advantageous in several processes such as biodegradation of environmental pollutants, promotion of plant growth and sustenance of the microbial balance with respect to human physiology, it may also pose colossal obstacles in several clinical and industrial set-ups. The bacterial agglomerates encased in the matrix of extracellular polymeric substances (EPS) lead to tenacious microbial infections; thereby presenting considerable medical exigencies. They are recalcitrant to antibiotics and host defenses; making the therapeutic treatments strenuous and expensive. Biofilms may indirectly cause dissemination of pathogens and persistent human infections representing serious public health concern. They are also responsible for product contamination, decline in heat transfer efficacy, rise in fluid frictional resistance and allied equipment damages as well as blockages and corrosion of metallic pipes posing sizeable economic problems.[9]

Some extracellular enzymes have the ability to detach this biofilm exo-polysaccharide cast from the cell surface; illustrating the suitability and advantages of their utilization as anti-biofilm agents to eliminate biofilms. Quorum quenching refers to the process or mechanism of disturbance and inhibition of quorum sensing of the organisms by such extracellular enzymes through disruption of their signaling or signal reception properties and induction dynamics during bacterial growth. Effectual biofilm degradation by quorum quenching enzymes (QQE) is hypothetically suggestive of plausible quorum sensing inhibition (QSI) pathway. Such biofilm removal strategy might as well be used as a promising approach towards the improvisation of treatment of multidrug-resistant microbial septicemia. Though more research is required to be done about the involvement of quorum sensing in biofilm formation, maintenance and dispersal, quorum sensing inhibitors (QSIs) have been projected as fairly reasonable antibiofilm agents.[10] Several QSIs have been identified by screening various chemical libraries of both natural and synthetic origin.[11]

1.4Juice Clarification

Rising demand for fruit juices has led to a concomitant increase in their production in recent years.[12] Fruit Juice clarification is a commercially significant bioprocess that enables the breakdown of a semistable emulsion of colloidal plant carbohydrates supporting the insoluble cloudy matter of freshly pressed juice thereby dropping its viscosity and modifying the opacity of turbid juice to an open splotchy look.[13] It is one of the most important techniques to increase the qualitative and quantitative characteristics of any juice before its commercialization, thereby improving its consumer acceptance and marketability.

The raw juice consists of water-soluble and insoluble components composed of fibrous matter, proteins, gummy matter, etc. that tend to precipitate with the passage of time during their storage, leading to sedimentation, thus lowering the product value.[14-15] The turbidity, viscosity & cloudiness in juice are mainly due to the polysaccharides viz. cellulose, hemicelluloses, pectin & lignin.[16] The enhancement of juice clarification can be achieved by the addition of cell wall degrading hydrolytic enzymes which disintegrate the fruit pulp to increase the juice yield.[17]

The present investigation involves testing of clarification of *Mangifera indica* is a tropical, indigenous, perishable seasonal fruit which is widely acclaimed for its taste, succulence and exotic flavor. It is rich in pectin, sucrose, glucose, maltose, vitamins and minerals. India is a major producer of mangoes and clarified mango juice is in huge demand for its established nutritive value and taste.[18]

1.5 Rationale

Inactivation and elimination of biofilms pose huge challenge in the industrial settings. Only inactivation of the attached biofilms without their removal would impart an optimal habitat for supplementary adhesion and growth, leading to a composite maze. Owing to the limitations of different chemicals applied for biofilm removal, replacement of chemicals with enzymes becomes imperative.[19] Moreover; microbial resistance to biocides is the primary motive for researching alternative biofilm control strategies. Enzymes are advantageous due to their high selectivity and capability of disrupting the structural stability of biofilm EPS matrix.

Initially evolved for utilizing the surplus stock of fresh fruits, the conversion of fruits to fruit juices and their processing has been steadily entrenched as a vital industry.[18] Chemical treatment of fruit juices is particularly slow and requires comprehensive downstream processing to obtain the clarified juice.[20] In contrast, enzymatic juice clarification reduces astringency in taste while improving the yield as well as physical quality characteristics such as color, viscosity, turbidity and filterability.[21]

Pectin-rich juices like that of mango, warrants the treatment with commercial pectinolytic enzymes for efficient clarification.[22] This is a major factor that contributes towards an increase in production cost to the juice processing industry. Hence microbial enzymes from their low-value substrates are preferred by fruit processing industries.[15]

The escalated cognizance of the applications of microbes in industrial bioprocesses has solicited the development of newfangled commercially rewarding biological products in various spheres. Hence, microbes from diverse exotic environments are being screened for specific properties expecting to result in new process applications.[23]

In this connection, the present study was attempted to chemically characterize protease enzyme extracted from cultivable microbial consortia screened from Indian mangrove swamplands and check its application for quorum sensing g inhibition and clarification of juice extracted from *Mangifera indica.*The present study is valuable in evolving an additional approach for the both the imperative industrial bioprocesses.

2. MATERIALS AND METHODS:

In the present investigation, isolates from sediment samples were screened for their protease production efficiencies. For this, twenty soil and water samples labeled as B_1-B_{20} were collected from marshy sediments of mangrove areas in Panjim, Goa. Latitude and longitude with water depth were measured for the sampling station. The physicochemical parameters like pH, temperature, dissolved oxygen and conductivity of sampling site were also measured.[24]

The organisms were isolated using spread plate method and their colony morphological characteristics viz. shape, size, color and consistency were studied.[25] All 20 bacterial isolates were screened and checked qualitatively for their proteolytic activity by spot inoculation on casein and gelatin agar platesusing standardized microbial assay.[26] The media was incubated at room temperature for 24h. The plates were stained with 5% Trichloroacetic acid (TCA) and observed for clear zone on turbid protein precipitated background. Clearing of milk protein around the colony was observed because of utilization of casein by the organisms.

Diameter of zones was measured and the values were noted for all 20 isolates. However, B_2& B_3 isolates manifested persuasive protease production proficiencies. 16S rRNA sequence analysis ensued their homology to Bacillus sp.[27] Further, bulk production of the enzyme sample was carried out from the most proficient B_3 isolate. Isolation and downstream processing involved three steps namely: [28]

- Bacterial Fermentation by shake-flask method
- Cell harvestation by centrifugation
- Microfiltration

After the production step, the filtered supernatant was concentrated 10 fold through Tangential flow filtration (TFF) process instead of conventional concentration methods and utilized for further investigations. Filtration is a pressure-driven seggregation process employing the membranes for partitioning of components present in a suspension depending on their relative size and charge ratio. In Direct Flow Filtration (DFF), the fluid is directly made to flow towards the membrane under optimum applied pressure while in Tangential Flow Filtration; the fluid is tangentially pumped along the membrane surface. The term tangential refers to the direction of fluid flow relative to the membrane.[29]

With respect to the characteristics influencing culture conditions, productivity and properties of the sample, it was considered sententious to characterize the sample through media variation studies,[30] process optimization,[31]Stability studies,[32] Spectroscopic assay[33] Kinetic parameter evaluation,[34] Inhibition studies[35] and Electrophoretic analysis.[36]

In application studies, TFF concentrated protease from B_3isolate was checked for its quorum sensing inhibition or quorum quenching ability and also employed for clarification of mango juice to check the nutritional and physical quality post-treatment.

2.3 Biofilm Degradation

We evaluated our sample; an extracellular neutral protease produced by *Bacillus safensis* under shake flask fermentation conditions, for its capability to target and degenerate the biofilm produced by a bacterial culture. Quantification of biofilm removal was done using crystal violet assay as described below.[37-38]

Loopfull of *Bacillus subtilis* culture was inoculated in 2.0 mL of appropriate broth and the tubes were incubated for 24h at 37°C. 1.0 mL of the three-fold diluted enzyme was added and the tubes were again incubated for 30 minutes at 45°C. The broth was removed by pipetting; 2.0 mL of water and 4.0 µL of crystal violet were added and further incubated for 15 minutes at 45°C. The crystal violet solution was pipetted out and the tubes were rinsed twice with distilled water. 1.0 mL of ethanol was added and absorbance was checked at 595 nm.

Following formula was used to obtain % degradation:

$$\% \text{ degradation} = \frac{\text{Absorbance of sample}}{\text{Absorbance of buffer}} \times 100$$

2.2 Juice Clarification

For testing the application of TFF concentrated protease from B_3 for juice clarification, fresh and ripe mangoes (*Mangifera indica*) procured from the local market were peeled, deseeded and grounded in a blender using distilled water in 1:1 (w/v) ratio so as to obtain homogenous pulp. 0.5 mL of mango pulp was diluted to 1:2 by buffer pH 7.0.[18] 0.5 mL of the three-fold diluted enzyme was added to the diluted pulp. The reaction mixtures were incubated at 60°C for 4h. Protein concentration was estimated by the standard assay system i.e. Bradford method at the beginning of the reaction (Initial) and after 4h of Incubation (Final).[39]

Following formula was used to calculate the percent degradation:

$$\% \text{ degradation} = \frac{\text{(Initial Protein Conc. - Final Protein Conc.)}}{\text{Initial Protein Concentration}} \times 100$$

3. RESULTS AND DISCUSSION:

As reported in our earlier publication,[40] morphological studies showed that colony sizes varied from 1 mm to 8 mm. Colors of the bacterial suspensions ranged from cream, yellow, orange tolight brown while their consistencies were found to be either opaque or translucent.

The qualitative analysis depicted B_3 to be efficient protease producing bacterial strain followed by B_2. The results significantly indicated that B_3 exhibited maximum activity which is a major requirement for commercial protease production.

Molecular evolution approach i.e. taxonomical identification of the organisms further confirmed isolates B_2 and B_3as belonging to the genus Bacillus.

Growth media optimization by media variation studies using six different media showed that the one containing specific protein substrate like gelatin gave the highest activity. Therefore, the sample obtained from gelatin media was used for further studies. Growth media pH and temperature optimization studies revealed that the optimum pH for growth medium was 7.0 and optimal temperature was 40°C. Enzyme pH optima studies revealed that maximum activity was exhibited at pH 7.0 indicating neutral nature of the enzyme. Temperature optimization studies showed maximum activity at 60°C, hence the sample was classified as thermozyme. The activity at extreme conditions of pH and temperature (80°C and pH 10) reduced to negligible.[41]

Qualitative spectroscopic studies revealed effective utilization of casein substrate by B_3 extracted sample. Kinetic studies showed lower Km values indicating higher affinity for the substrate.

Electrophoretic analysis indicated high molecular weight of the enzyme sample.

Inhibition studies showed that the salts Sodium chloride, Calcium chloride and EDTA tested for their effect on protease activity had inhibitory effect. Cobalt chloride showed activation at 10 mM concentration which changed to inhibitory effect above 20 mM concentration, as reported in our earlier publication.[42]

3.1 Biofilm Degradation

The extracellular neutral protease produced by B_3 isolate was tested byCrystal violet assay as described in the section Materials and Methods.It was found to be quite effective in biofilm degradation. Positive control Proteinase K showed 44.5% degradation while B_3extracted enzyme showed 90.5% degradation of the biofilm **(Refer Table1, Fig. 1).**

Johasen, Falhot and Gram (1997) reported that the enzyme composite (protease, amylase and pectinase) synthesized by the fungal strain, *Aspergillus clavatus* MTCC 1323 degraded the biofilms of *Pseudomonas aeruginosa*, *Bacillus subtilis* & *Staphylococcus aureus*.[43]

Leroy and co-workers (2008) evaluated the biofouling potential of few commercially pertinent enzymes on the adhesion of marine bacteria.[44]

Similar results have been reported by Molobela and group (2010) who studied the combined effect of protease and amylase for degradation of biofilm produced by *Pseudomonas fluorescence*.[45]

Bholay *et al*. (2012) reported that the enzyme expressed by *Bacillus pumilus* and *Staphylococcus auricularis* could efficiently degrade 86% and 50% of biofilm respectively.[38]

Meireles and co-workers (2016) in their review on quorum sensing enzymes have also reported that proteases are more effective in removing biofilms on glass wool or borosilicate glass surfaces.[11]

Watters *et al*. (2016) tested the antibiofilm efficiency of α-amylase, bromelain and papain enzymes against in vitro biofilm model of *Staphylococcus aureus*. They results showed that all of them significantly reduced the biomass within 24h; consequently they can be used as effective means of eradicating biofilms.[46]

Kumari and group (2018) applied thermophilic protease for degrading the biofilm of *Pseudomonas* and *E. coli* and demonstrated that the combination of proteolytic enzyme and polysaccharides was successful in biofilm removal or detachment by effectively dissolving mucupolysaccharides in biofilm.[47]

Asafor (2018) reviewed the research reports centered on the natural compounds with anti-QS potential.[48]

Fong *et al*. (2018) provided an outstanding application strategy for bacterial quorum sensing disruption by stimulating the combination effect of quorum quenching enzyme (QQE) and quorum sensing inhibition (QSI) in blocking the bacterial QS and validated their experiments with respect to *Pseudomonas aeruginosa* QS circuits.[49]

Anabela and Manuel (2019) in their review on bacterial quorum sensing systems and signal molecules emphasized the remarkable biological potentiality of marine species, ignorance towards the exploration of marine habitat and the application of marine bacteria or their metabolites as quorum sensing inhibitors.[50]

3.2 Juice Clarification

Optical densities of the tested samples showed the effect of enzyme on the clarification of a local variety of mango juice. In the present experiment, no visual difference was obtained in mango juice clarification. Careful protein estimation, however, revealed 18.1% of protein degradation from mango pulp by B_3extracted sample and 60.3% of protein degradation by the control enzyme Proteinase K. Thus, the percent clarification obtained for the test sample was 18.1% and that for the positive control was 60.3%. **(Refer Table 2, Fig. 2)**

Enzymatic juice clarification has been reported by researchers' viz. Sreenath and Sudarshana (1994) who investigated the mango juice liquefaction with a commercial enzyme mixture.[51]

Singh, Mayer and Lozano (2000)[52] have investigated the most favorable liquefaction conditions for retrieving mango pulp (Keitt variety) with low viscosity and good filterability, using a commercial enzyme mixture in order to assess the changes in the physicochemical parameters notably color, clarity, aroma, viscosity and serum yields during the liquefaction.

Ndiaye *et al*. (2011) optimized the process parameters for clarification of natural cloudy mango juice by using cellulolytic and pectolytic enzymes.[53]

Kumar and co-workers (2013) studied the clarification of mango juice by cellulase extracted from *Aspergillus niger*.[15]

Sharma *et al*. (2014) have reviewed the enzymatic extraction and clarification of juices from different fruits.[21]

Jori *et al*. (2015) demonstrated multienzymatic clarification process of blended Pineapple and Mango pulp.[54]

Anuradha and group (2016) reported the use of

Polygalacturonase expressed by *Aspergillus awamori* MTCC 9166 for clarification of mango juice and established the enzymatic conditions for clarification by optimizing of system parameters viz. incubation time, temperature and enzyme concentration to obtain maximum juice yield.[18]

Kunkolol *et al*. (2018)[55] have reported the process optimization for multienzymatic clarification of Totapuri Mango Pulp with respect to temperature, time, yield, color and clarity to make the process suitable for practical application to the mango juice processing industries.

Table 1: Percentage Degradation

Sample	**Absorbance (595nm)**	**% degradation**
B_3 Protease (Test sample)	0.114	90.5%
Proteinase K (Positive control)	0.666	44.5%
Buffer (Negative control)	1.00	0%

Fig. 1: Crystal Violet Assay

Sample **Control** **Buffer**

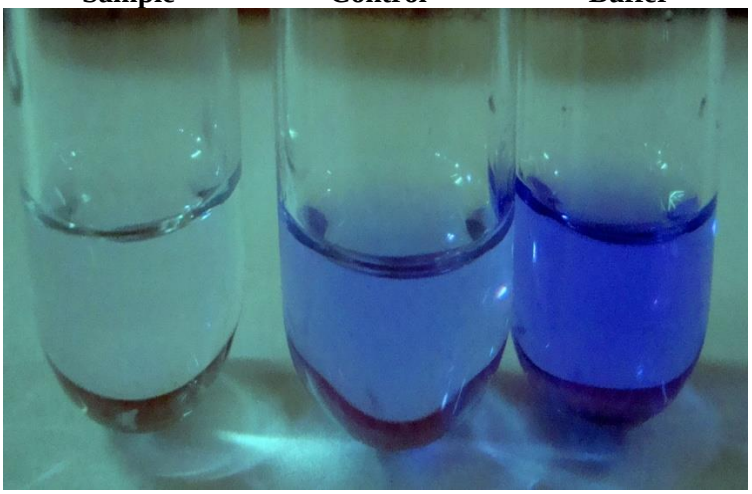

Table 2: Percentage Clarification

Sample	Initial Protein Concentration (mg/mL)	Final Protein Concentration (mg/mL)	Percent Degradation (%)
B_3 Protease (Test sample)	15.2	12.45	18.1%
Proteinase K (Positive control)	20.1	7.98	60.3%
Buffer (Negative control)	16.0	16.0	0.0%

Fig. 2: Bradford Assay

Sample **Control** **Buffer**

4. CONCLUSION AND BLUE PRINT FOR FUTURE:

This paper reports the characterization and application studies of a neutral protease extracted from Bacillus sp. From the present study, it is evidenced that Bacillus sp. isolated from estuarine ecosystem can be harnessed as a potent microbial source for large scale production of industrially imperative protease enzyme under optimized nutritional environmental conditions.

Investigations on the catalytic and non-catalytic domains of the vast proteolytic landscape to establish these blunt aggressors of protein demolition as efficient executioners of different chemical reactions form the future scope of the project. This specifically includes:

- Accelerated ageing studies
- Application studies for other industrial bioprocesses viz. Albumin degradation, Keratinase activity, Antibacterial activity & Wash performance analysis
- Application studies as biocatalyst for chemical transformations, particularly C-C bond formations

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7. CONFLICT OF INTEREST:

The author also declares no conflict of interest.

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