Molecular characterization of pectinase producing innovative bacterium, Aeromonas guangheii, isolated from the gut of fish, systomus sarana, RSM optimization for its enzyme production, the cloning and expression analysis of exo-pelA-gene and the recombinant protein purification

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Abstract

During the present research, 11 gut bacteria were isolated from the fresh water fish, Systomus sarana (General name: olive barb) and upon screening, the strains produced extracellular pectinase enzyme. Among them, the SS6 strain was found to produce a high quantity of 208.731 U/mg pectinase. Through molecular characterization the SS6 strain was identified as Aeromonas guangheii. In this study, a group of controlled experimental factors were investigated to optimize through the response surface methodology with a Box-Behnken design. The optimal conditions were found to be; 2.11% of maltose, 2.20% of yeast extract, 6.5 of pH, and a temperature of 27.3 ºC at 32-h incubation. Under the above conditions, the activity of pectinase production was enhanced to 371 U/mg. The purified pectinase's molecular weight was determined to be ~50 kDa (by 10% 2-D PAGE). The FT-IR analysis of the degraded pectin-products revealed the presence of six functional groups. Totally, nine peptides were identified from the purified pectinase enzyme through the MALDI-TOF-MS analysis and MASCOT tool was used to get the mass spectrum of the peak at 2211 of peptide that indicated the reference pectinase protein. The referenced gene primer (pectate lyases) was PCR amplified and their nucleotide sequence was analyzed. The exo-pelA gene was cloned in pREST vector, that was over expressed in Escherichia coli BL21. The ORF encoded for a protein of 425 amino acids with a predicted molecular weight of ~50 kDa. The present findings underline the potential of the fish-gut microbes as a source of biotechnologically important enzymes.

Introduction

Microbes are considered to be a rich source of enzymes and hence the exploration of their extracellular enzymatic activity is an important step for the production of industrial enzymes from bacteria, fungi, yeast, plant and animal sources. The importance of microbial enzymes for the production of food, alcoholic beverages, drugs, vaccines and in health supplements have been highlighted by earlier researchers (Dalvi et al. 2007; FAO. 2014; Hoondal et al. 2002). Some recent researchers have focused on the use of gut flora and their associated enzyme towards the improvement of innate immune response, nutrient metabolism and for proliferation of epithelial cells in cultured-animals (Sasmal et al. 2015). The fish-gut bacteria are considered to be a dynamic source of hydrolytic enzymes that have the potential applications in many industrial sectors viz; aquaculture, biotechnology, food, pharmaceuticals, detergents, paper and textiles (Mondal et al. 2010). Ant the gastro-intestinal (GI) of the -bacteria of freshwater fishes are of particularly important in this regard because the microbial enzymes from the aquatic animals are renowned for their extraordinary chemical and metabolic characteristics (Debashish et al. 2005)

The pectinase enzyme of the pectate lyase – group is a pectin depolymerizing one, as it can cleave α-1-4 galacturonosidic linkages of polygalacturonic acid (PGA), through the transamination mechanism and which results in the generation of unsaturated bond at the non-reducing end of the oligonucleotides (Klug-Santner et al. 2006) As such, the pectin is composed of a group of polysaccharides like galacturonic acid (GalA) that are in the form of covalently linked structural domains viz; homogalacturonan (HG), xylogalacturonan (XGA), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) (Mohnen et al. 2008). The pectinase is considered to be an important enzyme as it is being used in pharmaceutical industry (for drug delivery) and textile industries and interestingly, as a dietary supplement for humans, because of its possible cholesterol-lowering effect (Agüero-Chapin et al. 2009; Khan et al. 2013; Morris et al. 2010). Of late, some investigations have been made on the synthesis, characterization and the utilization of microbe-based enzymes viz; purification, structural–functional characteristics, and industrial applications (Sharma et al. 2013). An earlier investigation in the Erwinia chrysanthemi strain has resulted in the identification of nine extracellular pectate lyases viz; exo-Pel (PelX) (6) and 8 endo-Pels (PelA, PelB, PelC, PelD, PelE, PelL, PelZ and PelI) (Shevchik et al. 1997). Hugouvieux- cotte-pattat et al. (1996) have detected over 60% of the Pel activity in the supernatant of Azospirillum irakense (in its mid, late-exponential and stationary phases of culture) and which indicated the transport of degrading enzymes to the extracellular medium.

In view of the industrial - importance of the pectinase enzyme and the innovative and eco-friendly nature of its production, the objective of the present work was to investigate the production of extracellular pectinase enzyme from the gut bacteria of *Systomus sarana* (its commonly available and edible freshwater fish). And the response surface methodology was used to ascertain the optimum condition-requirement (for the maximum enzyme production) proteo-genomic characterization and exo-pelA gene expression (into *E. coli* BL 21) were also carried out.

Materials And Methods

Bacterial isolation, their screening and enzyme assay

Totally 20 fishes were collected from the Cauvery River at the Stanley Reservoir, Salem District, India (21°43.232'N, 87°48.884'E). The collected fish was identified as *Systomus sarana* by following the keys provided in the Manual of Jayaram et al. (2010). The fish gut contents were homogenized, and serial diluted up to 10^{-5}. The spread plate technique was performed on sterilized tryptone soya agar-medium that composed of Sodium chloride-5.0 g/L, Dextrose-2.5 g/L, Dipotassium hydrogen phosphate-2.5 g/L and Agar-15.0 g/L (Hi Media, Mumbai, India). The isolate's growth condition was 37°C, on nutrient agar media plate and finally 40% glycerol stock was made for the long-time storage at-20°C. For the primary screening of pectinase enzyme production, the isolates were grown on pectin-agar media (5 g/L pectin, 5 g/L tryptone, 2.5 g/L yeast extract, 5 g/L NaCl, 15 g/L agar; pH 7). By following the well diffusion method, 100 µl of cell-free supernatant was loaded on a pectin agar well plate
(of diameter 5 mm) and the plates were incubated at 37°C for 24 h. The amount of the enzyme produced was then calculated, and expressed as enzyme intensity by following protocol of Hankin et al. (1971) (EI) = (colony diameter + halo zone diameter) / colony diameter. The pectinase enzyme activity was determined using 3, 5-dinitrosalicylic acid method of Miller et al. (1959). A unit of pectinase activity is defined as 1 µmol of galacturonic acid released per minute.

Molecular characterization

Bacterial genomic DNA (gDNA) was isolated according to the phenol: chloroform method (Sambrook et al. 2001). The purified DNA was subjected to PCR amplification using a pair of 5'-AGAGTTTGATCMTGGCTCAG-3' and 5´-AAGGAGGTGATCCAGCCGCA-3´ forward and reverse primers for 16S rRNA gene amplification and sequencing. The phenotypic level identified gyrB F (5'-GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYGA-3') and gryB R (5'-

AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGTCAT-3'). A total volume of 25 µL (2x dream taq master mixed) was used for PCR reaction. The PCR program consisted of an initial denaturation step at 95°C for 5 min followed by 30 rounds of temperature cycling comprising denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1.30 min, followed by a final extension at 72°C for 7 min and then cooling at 4°C. The sequences have been deposited in the National Center for Biotechnology Information (NCBI) Data Bank Accession number; MF800949. The phylogenetic and molecular evolutionary analyses were conducted by using the software MEGA by the neighbor-joining method (Tamura et al. 2013).

Response Surface Methodology (RSM)

Response surface methodology was performed as per the protocol of Ramesh et al. (2013), with slight modification. A five-factor Box-Behnken Design contained 46 experimental runs with three replications (**Table 1a**). It was also used to optimize the independent variables, like, pH, temperature, carbon sources, nitrogen sources, inoculum size and incubation day. The modeling and statistical analysis was used Stat-Ease Inc., Minneapolis, software. Plackett-Burman (PB) design: Totally, 12 factors viz; glucose, maltose, sucrose, galactose, lactose, mannitol, beef extract, peptone, yeast extract, $NaNO_3$, NaCl and KH_2PO_4 were considered (Plackett et al. 1946).

Effect of various parameters on pectinase production

The effective isolate was inoculated into the pectinase production medium at the optimum ranges of distinct parameters viz; pH (3-10), temperatures (10-45°C) and incubation time (0-60 h) and incubated under rotary shaking at 150 rpm. The effect of six carbon sources each to 1% and six nitrogen sources each to 1% on the production of pectinase was carried out (Kumar et al. 2011).

Extraction and purification of protein

The isolated overnight culture broth (at the optimum condition) was centrifuged (10,000 rpm; 4°C; 20 min), the cell-free supernatant was collected and an 15% Ammonium per sulfate was added to precipitate the protein. And then the protein was purified using gel chromatography, where 20 ml of Sephadex G-100 solution was packed into a glass column (35×1.5 cm, 60 ml of bed volume). The 10 ml of precipitated protein-loaded column was eluted using the Tris-HCl buffer (10 mM, pH 7.5). Then, 1.5 ml volume of fractions was collected separately and stored at −80°C for further analysis.

Two-Dimensional (2-D) PAGE analysis

The purified protein (20 µl) was loaded onto 12% SDS-PAGE with a standard marker (GENEI, Bangalore, India) and electrophoresis was performed as per the protocol of Laemmli et al. (1970). Further 2D analyses, the protein samples were sent to Sandor life science Pvt. Ltd. (Hyderabad). The 2-D gel was analyzed by an in-silico method based on the isoelectric point (pI) and the molecular weight of separated proteins. The 2-D gel protein spot was analyzed by using the ExPASy tool.

FT-IR and HPLC analyses

ART model FT-IR spectrophotometer was used at the wavelength of 4000- 400 cm^{-1} and the resolutions of 4 cm^{-1} were averaged according to Cakmak et al. (2006). The enzyme fraction sample was investigated by HPLC using Shimadzu LC solution 20 AD, Japan and SPD 20 A, having a reverse phase water guard Column: Symmetry LCGC C18 (5 µm,4.6×250 mm) and Hamilton microliter syringe using an injection volume of 20 µL and detection was made at 254 nm by UV detector at 28ºC. The HPLC mobile phase comprised of methanol and water (50:50). $Empower^2$ software was used to analyses the data.

Charaterization of the Mass Spectroscopy of extracted protein and homology modeling

Mass spectra of the digestion mixtures were acquired in a 4800 MALDI-TOF-MS instrument (Applied Biosystem) in reflector mode and were externally calibrated using a mixture of peptide standards by following the protocols of Patterson et al. (1995). Collision-induced dissociation MS/MS experiments of selected peptides were performed. A NCBI database search with peptide m/z values was made by using the MASCOT search tool (URL http://www.matrixscience.com) for the identification of tryptic maps of protein identification. Homology modeling was performed with SWISS-MODEL a homology-modeling server as described by Bordoli et al. (2009).

PCR amplification of exo-pelA gene

PCR using the exo-pelA gene primer *pelA* f (5' GATAAGGATCCGATGCAAGACAGCGACGTG 3') and *pelA* r (5' GCTTCGAATTCCGCACAAAGAGAAAGGAAT 3') in a 30-µL reaction volume that contained 15 µL of 2x dream taq master mix (Thermo), 1 µL genomic DNA (50 ng/µL), 1 µL of each primer (20 pM) and water was used. The PCR program consisted of an initial denaturation step at 94°C for 3 min followed by 35 rounds of temperature cycling comprising 1 min at 94ºC for denaturation, 30 s at 57ºC for annealing, 40 s at 74ºC for extension, and 7 min final extension.

DNA/pREST vector digestion and ligation

The pREST plasmid (~2.7 Kb) *E. coli* strain: For the isolation of plasmid genomic DNA, the protocol of Sambrook et al. (2001) was followed. An aliquot of DNA sample was mixed with 2 µl of 1 x restriction buffer (5 µl) and the volume was made up to 30 µl with ddH_2O. The reaction was started with the addition of the enzyme *EcoRI* (1 µl), *BamHI* (1 µl) and incubated at 37°C for 2 h. Incubation at 70°C for 3 min terminated the reaction. The cocktail mixtures (30 µl reaction) contained 10x T4 DNA ligase buffer (4 µl), vector (4 µl), insert gene (3 µl), T4 DNA ligase (5 µl), ATP (1 µl) and ddH_2O 13 µl. The mixtures were added into the microtubes and placed on ice, and then the samples were incubated at 4ºC for 16 h.

Preparation of competent cells and transformation

A single colony of *E. coli* (DH5α) culture was inoculated in 5 ml of LB medium (with AMP) and incubated at 37°C for 16 h. And 1.0 ml of 16 h grown culture was added to 100 ml of LB medium in a 250 ml conical flask and incubated at 37°C for 16 h. For the competent cells preparation ($CaCl_2$-treated) and transformation (heat shock treatment), the method of Sambrook et al. (2001) was followed.

Gene expression analysis and recombinant protein purification

An LB medium containing AMP was used (pH 7.0) for pectinases production. A modified basal synthetic (BS) medium was used for recombinant *exo-pelA* production in a 250 ml fermenter. The glycerol feeding solution was used for high-cell-density cultivation (HCDC) of *E. coli* (BL21). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was filter-sterilized and added to the bioreactor as an inducer. The recombinant protein was purified by Sephadex G 100.

Effect of metal ions on pectate lyases

20 ml of sterile production medium was prepared in different conical flasks. Each flask was amended with effective metal ions such as $Ca2^+$, $Mg2^+$, $Co2^+$, $Cu2^+$, $Fe3^+$, and $Mn2^+$, individually. The final concentration of the enzyme substrate system was 5 mM. The reaction mixture was terminated by adding 2 ml of 3, 5-dinitrosalicylicacid reagent and then the enzyme activity was assayed.

Results

Pectinase enzyme production

In total, 11 bacterial strains were isolated from the freshwater fish gut-tissue and they were named as SS1 - SS11. All the isolated strains upon primary screening revealed the formation of clear zone around their colonies, confirming the production of pectiase enzyme. During the screening of SS6 strain, initially showed an enzyme index with the highest zone of clearance of 4.7 mm **(Fig. 1A &1B).** The strain SS6 showed the highest enzyme activity of 208.737 U/ml during secondary screening (**Fig. 1C**). The SS6 strain was selected as efficient pectinase-producing bacterium and that was used for further studies.

Molecular characteristics of the effective bacterial strain

The effective strain SS6 was found to be gram-negative and a rod-shaped bacterium. The amplified product had a 1400 bp length on agarose gel electrophoresis. During comparison with the database of NCBI, the present sequences showed a 99% similarity with *Aeromonas guangheii.* The phylogenetic tree was constructed for *A. guangheii* (**Fig. 2A**) by comparing with strains from GenBank with the highest similarities. The *A. guangheii* showed more than 99% of homology to the nearest neighboring sequences and at least 98% homology to the closest type strain sequences, thus supporting the taxonomic annotation to the minimum genus level.

Response Investigation findings

The combined effect of the parameters was studied at an incubation time of 3 days with shaking incubator (120 rpm). The experiment was conducted using CCD, and the experimental and predicted results for pectinase enzyme activity are given in **Table 1a & b**. The optimum growth condition for the production of pectinase (371 U/mg) was achieved by using maltose (2.11%), yeast extract (2.20%), pH (6.5), temperature (27.3°C) and incubation time (32 h).

Optimized condition for pectinolytic activity

A three-dimensional (3D) response surface curve was plotted to understand the interaction effect of the three components required for maximizing the yield of pectinase enzyme production. The collective effect of the initial temperature-pH and incubation-maltose; incubation-temperature and temperature-yeast extract on pectinase enzyme production is shown in **Figs. 3A& B.** The maximum pectinase enzyme production occurred at pH-6.5 (**Figs. 3A- a, b)**. The combined effect of the temperature yeast extract; incubation time-temperature and temperature-maltose exhibited a maximum pectinase enzyme production at the temperature of 27.3°C **(Figs. 3A- c, g).** The combined effect of maltose-yeast extract; maltose-pH and incubation time-maltose inhibition/composition resulted in highest production of enzyme at 2.11% maltose and combined effect of temperature-yeast extract and pH- yeast extract on the enzyme production indicated that yeast extract at 2.20% (**Figs. 3A- f, h)**. **Figure 3A- e, j** represents the 3D plots of the collective effect of incubation time-maltose and incubation time-yeast extract on pectinase enzyme production, with a maximum at the incubation time at 32 h. The impact of pH on the production of pectinase enzyme was examined over a broad range of pH 3.0-10.0. The optimal pH for the production of pectinase was slightly acidic (6.5), and when the pH was gradually increased to alkalinity (up to 10), the pectinase activity was relatively decreased. Adequacy model, it is given proceeding with analysis and optimization of the fitted response surface would likely give poor/ misleading results. By constructing a normal probability plot of the residuals, a check was analyzed for the normality assumption as shown in **Fig. 3B.**

Statistical Analysis

A quadratic model in comparison to a cubic model is suggested because it has a higher value of adjusted R^2 and predicted $R^{2.}$ **Table 1c** shows the outcomes of the second-order response surface model fitting in the form of ANOVA. The lack-of-fit of the model is not significant with an *F*-value of 0 and Prob > *F* of 1.0 indicates the experimental data were accurately fitted by the model. Moreover, the goodness-of-fit of the model was tested by analyzing the coefficients R^2, Adj R^2, and Pred R^2. In this case, R^2 is 0.993, which indicates a high correlation between the experimental and predicted values and demonstrates that only 1.12% of the total variation was not explained by the fitted model. The Pred R^2 of 0.994 is in reasonable agreement with the Adj R^2 of 0.975. The obtained ratio of 35.664 indicates an adequate signal. The coefficient of variation is 2.721%. **Table 1d** shows the *P*-value and *F*-value of the variables included in each model. Values of Prob. > F less than 0.05 indicates model terms are significant. The regression model prepared to predict the production of pectinase enzyme (enzyme activity = 315.54 U/mg) by the isolate *Aeromonas guangheii.* Pareto chart demonstrated that the two factors *viz*; maltose and yeast extract, affected

the pectinase production positively **(Fig. 3C-a, b).** The Pareto chart also demonstrated the effect of significant parameters, and most vital effects are usually closer to, or extend past, the red colored line given in the chart.

SDS-PAGE analytical results of pectinase protein

The 1-DE analyses showed different molecular weight protein banding patterns that ranged between 180 and 20 kDa, where the most intense protein band was located at 50 kDa **(**Fig. 4A- a) and the protein variability was indicated by 1-DE analysis which was then confirmed by 2D-DIGE. The isoelectric points (pI) of isolated protein spots were observed on the 2D-DIGE gel, and the predicted protein spot was found at pH 6.5 **(Fig. 4A-b).**

The components of pectinase protein by FT-IR & HPLC analyses

Six functional groups (range of wavenumbers: 3359, 2361,1648,1576,1415, and 1044 cm^{-1}) were found from the extracted pectinase protein. By comparing with the existing literature, functional classes were found as 1º, 2º amines, amides (N-H stretch), nitriles (C=N stretch), 1º amines (N-H bend), aromatics (C-C stretch (in-ring)), alkanes (C-H bend) and aliphatic amines (C-N stretch) **(Fig 4B-a)**. The HPLC analysis of enzyme showed 11 peaks with the 2 major peaks at the retention time of 5.718 (19.41%) and 6.111 (77.33%) and the nine minor peaks at the retention times of; 3.799, 8.486, 10.637, 17.604, 20.571, 22.859, 25.021, 27.208 and 28.547 min (**Fig. 4B-b**).

Spectral features of 50 kDa protein

The forms of *Aeromonas guangheii* (as the 50 kDa protein bands from the SDS-PAGE) were digested with trypsin and the resultant peptide mixtures were analysed by MALDI-TOF mass-spectrometry (**Fig. 5A)**. As expected, almost identical mass-spectra were obtained for proteins. Resulting from trypsin specific cleavage and matching the *Aeromonas guangheii* amino acid sequence, twenty-four peptides (with the m/z values in the range of 914.5–2705.1) were found (**Fig. 5B and Table 2)**. The specific matching peptides for the 50 kDa enzymes covered 70% of the whole sequence, respectively (**Fig. 5C)**. Homology modeling was performed based on the pectinase amino acid sequence, it is considered to be the most accurate of the computational structure of pectinase protein and the predicted three-dimensional structure of a protein by ribbon and MESS model **(Fig. 6A).** We found to validate the model with 94.0% of 386 total residues in the core region, 6.0% of residues in the additional allowed region followed by the residue in the disallowed region through Ramachandran plot **(Fig. 6B).** In **Table 3,** the result displayed the good stereo chemical structure quality of the PGs model. This showed that in the refined model, the backbone dihedral angles phi (φ) and psi (ψ) occupied reasonable accurate positions in the three-dimensional model.

Features of the amplified exo-pelA gene

The effective pectinase enzyme producing strain SS6 was screened and it was identified to be a gram-negative, rod-shaped bacterium **(Fig. S1a, b).** The amplified exo-pelA gene product was found to be approximately 1250 bp in length on agarose gel **(Fig. S1c).** The 16S rRNA sequences were found in the NCBI database, and exhibited a 98% resemblance with the *Aeromonas guangheii pelA* gene. A phylogenetic tree was constructed based on the NCBI data base sequences (n=19) with our sequence and a total of 1849 nucleotide positions in the final dataset were sequenced by the neighbor-joining method (**Fig. S2).**

Expression of the exo-pelA gene in *E. coli* BL21 (DE3)

To examine the expression of the gene in a heterologous host, the expression vectors pREST were transformed into *E. coli* BL21 (DE3). The recombinants were cultured in LB medium followed by induction using IPTG. The pectinase activity of pREST reached its maximum after 3 h induction (10 U/mg) which was 4 times higher than wild strain *Aeromonas guangheii*.

Recombinant protein exo-pelA gene

The gene product was further verified in *E. coli* by SDS-PAGE. Staining with silver staining revealed one distinct band with a molecular mass of approximately 50 kDa, corresponding to the predicted molecular mass of the exo-pelA gene **(Fig. S3)**. The PCR amplified exo-pelA gene was cloned in pREST vector and insert was sequenced. The sequence results indicated that the cloned sequence contained an open reading frame (ORF) which started with an ATG start codon and terminated with a TAG stop codon. The ORF of endonuclease gene consists of 1236 nucleotides encoding a protein of 425 amino acids with a predicted molecular weight of 48.7 kDa **(Fig. S4)**. The pectinase assay of the transformed cells indicated a large and distinct clear zone around the colony and well in the pectin plate that confirmed the high pectinase activity **(Fig. S5a, b)**.

Enzyme activity, Effect of metal ions and HPLC analytical results

The purified negative pectinase and recombinant exo-pelA on pectate lyases (EC number as 4.2.2.9) activity was conducted and the efficient two fractions' activities observed were; 856 (U/mg) and 1203 (U/mg) in the presence of pectinase. The observed highest relative activities were; 143% and 137% in the presence of $MgCl_2$ and $CaCl_2$ metal ions, respectively. The lowest activity observed was in the presence of $FeCl_2$ metal ions **(Table 4).** The enzymatic reaction product was analyzed by HPLC **(Fig. S6).**

Discussion

During the present investigation, the fish-gut isolated innovative bacterial strain, *Aeromonas guangheii* has been successfully used for pectinase-enzyme production. Only very limited reports is available on the pectinolytic enzyme producing gut-bacteria of fishes, so far. Previously Mountfort et al. (1993) and Sasmal et al. (2015) have reported the possession of pectinolytic properties in bacterial species that were isolated from the gastro-intestinal tract of the fishes of the groups, Mullet and Tilapia. Interestingly, the presently isolated bacterial strain exhibited 76 % enzyme -activity **(Fig. 2B).** which is relatively 9-fold higher than the values recorded by the previous researchers. Hossain et al. (2020), have earlier reported only 24 % of pectinolytic activity by the

bacterium *(Aeromonas spp.)* isolated from the gut of Bombay Duck-fish. And most of the previously demonstrated enzyme-producing bacteria like *Bacillus pumilus*, *B. circulans* and *B. cereus* were isolated from the gut region of *Rohu* fish (Ghosh et al. 2002). The Presently isolated *Aeromonas* strain SS6 that produced pectinase enzyme has been characterized up to the species level and identified as *Aeromonas guangheii* - SS6. An earlier study on *Aeromonas*-Phylogeny by Bhowmick et al. (2018), specifically between the species *A. caviae* and *A. trota*, and between *A. culicicola* and *A. hydrophila* revealed the number of nucleotide difference of 102 and 79 respectively, (in relation to *gyrB* sequences).

Presently, the Box-Behnken design was implemented for a total 46 factors. Such an experimental design was earlier adopted to improve the reaction conditions for the production of a highly glucose tolerant β-glucosidase and for maximizing the enzyme yield by the bacterium *Paecilomyces variotii* (Job et al. 2010). The interaction of the most noteworthy parameters was further analyzed in RSM by referring to the similar earlier work of Hu et al. (2018). Presently the pectinase production-rate was more stable at pH – 6, which is similar to the previous report of Doğan et al. (2008), who have found more stable polygalacturonase activity at the pH of 6.0. However, the stability was significantly reduced at lower pH and the inactivation process was found to be faster at high alkaline pH values due to disulfide exchange (which usually occur under the alkaline conditions). This might be attributed to histidine residues with ionizable side-chains, which could increase the pH range by a net negative charge on the molecule during repulsion between the strands, which, in turn could have destabilized the hydrogen-bond structure of the enzyme, as reported earlier for *Penicillium viridicatum* at a pH of 6.0 (Silva et al. 2007).

Presently, the increase in temperature increased the pectinase production only up to a certain limit, after which the production rate was decreased. A maximum enzyme activity of *A. guangheii* was recorded at 27.3ºC. In an earlier temperature optimization experiment of Soares et al. (2001), for the production of all the endopolygalacturonase, exopolygalacturonase and pectolyase the highest activities were recorded at 28ºC, by the enzymes from the five *Bacillus* strains (viz., *Bacillus* Ar1.2, *Bacillus* M2.1, *Bacillus* Ega22, *Bacillus* Ega16, and *Bacillus* B1.3). Likewise, the acidic pectinase and polygalaturonate type of pectinase production were found in *Clostridium thermosacccharolyticum* at the optimum temperature of 30-40ºC (Van Rijsse et al. 1993). And Nagel et al. (1968) have reported that the enzyme production by the strain of *Bacillus* sp. was found to be relatively stable below the temperature of 30°C. Nitrogen is an essential constituent of amino acids, and it is an important element which is normally required for the growth of microbes and their production of enzymes. Presently, different organic and inorganic nitrogen sources were tried and the yeast extract supplementation was shown to enhance the pectinase production. Earlier researcher found that *Bacillus subtilis* SAV-21 bacterium purified with yeast extract supplementation revealed such an enhanced pectinase production (Kaur et al. 2017). In contrast to the present findings, a high level of production of pectinase in solid state fermentation condition was recorded in *Bacillus* sp., when supplemented with yeast extract (Rehman et al. 2012). During the present investigation, the maximum pectinase production was obtained through the maltose-supplementation. Earlier, pectinase production using batch fermentation by growing in a production medium containing 1.0% pectin as a sole source of carbon, utilized various sugars including glucose, maltose and galactose to produce acid pectin from *B. licheniformis* KIBGE-IB21 (Rehman et al. 2015).

Presently, the sugar group of alkanes (C-H bend) and aliphatic amines (C-N stretch) functional classes were identified from pectinase protein. Similarly, sugar functional groups were analyzed C-H and C-C stretching vibration in the FT-IR spectrum (Max et al. 2007). Subsequently, it is based up on the calibration of the peak intensities was found carbohydrate as glucose (1033 cm-1) and sucrose (995 cm-1), galacturonic acid in the fingerprint region (1200-900 cm-1) (Adina et al. 2010).

Kant et al. (2013) have earlier reported that the *Aspergillus niger* – produced Polygalacturonase enzyme with heterodimer of 69 and 34 kDa subunits, which is similar to the present findings. Some earlier researchers have reported the molecular weights (kDa) of the partially purified pectinase enzyme of different bacterial species *viz*; 31 for Streptomyces (Arijit et al. 2013) 66 for *Bacillus sp* (Giacobbe et al. 2014) and 89 for *B. cereus* (Bhardwaj et al. 2014). Earlier, Zhou et al. (2017) have reported on pectate lyase produced by *Bacillus subtilis* (BsPel). The MS/MS analyses of five peptides (Peptide2, Peptide4, Peptide7, Peptide9 and Peptide12) confirmed that they are unique peptides and the secondary mass spectrum of one representative P2 (3395.55 kDa). Saladino et al. (2005) have reported the sequence of two different proteins with a similarity of >25% with similar 3D structures. Gandreddi et al. (2015) have reported the template and similarity of the targeted sequences that had 42% and the 99.2% of amino acids in the favored region, confirming it to be a good model.

Henrissat et al. (1995) and Wang et al. (2014) have determined the pectate lyase activity of different bacteria through multiple sequence alignment analyses and their sequence identity of mature PelB gene was compared with 92% of Pel A & B from *Bacillus subtilis* subsp. subtilis str.168 80% of PelA from the *Erwinia chrysanthemi* EC16, 53% of PelA from Thermotoga maritima MSB8, and 32% of PelA from *Bacillus* sp. N16-5.

Presently, the exo-pelA gene expression in *E. coli* BL21 was confirmed via molecular mechanism for blue/white screening. It's based on a genetic engineering of the lac operon in the *E. coli* as a host cell combined with a subunit complementation achieved with the cloning vector. Comparing a cellulose with a pectinase, the purified recombinant enzyme had an activity of 0.50 ± 0.01 µM/min/ml, as determined by the carboxymethyl cellulose (CMC) assay at 60°C and pH 6. Whereas, the pectinase recombinant protein purified from *A. guangheii* resulted in a 3-fold collection have been increase in enzyme activity. In the present study, the maximum activity of the recombinant strain of exo-pelA gene, which codes for pectate lyase enzyme production, was found to be 1200 U/mL. Several earlier researchers have reported the maximum pectinolytic production by the Pel genes from *Bacillis subtilis* (142 IU/mL), *Bacillis pumilus* BK2 (76.8 IU/mL), and *Paenibacillus barcinonensis sp* (104 IU/mL) (Klug-Santner et al. 2006; Soriano et al. 2000; Soriano et al. 2006). Hence, purification and the expression effects of *AerPel*A gene were analyzed by SDS-PAGE (10%), and during which the whole protein of the positive clone (lane 2) exhibited a distinctive protein band at 150–15 kDa, (when compared to the negative control, Lane 1). The purified sample (Lane 3) revealed only one protein band. The apparent molecular weight was 50 kDa, which corresponded to its calculated 250 kDa protein marker. Earlier, some researchers have estimated the molecular weight of the expressed recombinant endoglucanase of *B. subtilis* that ranged between 40 and 70 kDa (Jung et al. 2010; Pandey et al. 2013). Ibrahim et al. (2015) have earlier reported molecular weights of the expressed proteins (performed using SDS- PAGE and celB, celC and peh products) that were found to be approximately 29.5 kDa, 40 kDa and 41.5 kDa, respectively.

Therefore, during the proteomic study of the presently expressed *pelA* gene, the nucleotide sequence has been translated with the protein sequence, where the molecular mass of the pectin lyase was determined as 45.7 kDa (by SDS-PAGE), which matches with the theoretical value of pI (5.2). Similar findings were

earlier reported from *A. irakens* by PelA, which revealed a molecular mass of 44.4 kDa and a pI of 6.2, at an optimum pH of 9.0 and an optimum temperature of 40°C Bekri et al. (1999). Previously, Wang et al. (2014) have reported the gene pelB that consisted of 1263 nucleotides and multiple sequence alignment with pectate lyase produced by the bacteria from metagenome-derived alkaline pectate lyase. They have found that PelB had the highest sequence homology with the pectate lyase family that encoded the mature protein (PelB) with 399 amino acids, which was expressed in *Escherichia coli*.

Out of the 11 fish gut bacterial strains obtained. Presently, only 9 forms have exhibited pectinolytic activity, with the highest activity was shown by the SS6 strain, which was subsequently identified as *Aeromonas guangheii*. The optimized parameters for the maximum enzyme yield by SS6 strain were, 6.3 pH, and 27ºC temperature with maltose **(2.11%),** and yeast extract **(2.20%)**. The characterization of SS6 strain showed its molecular weight to be ~50 kDa and its composition as glucose, fructose, sucrose, and galacturonic acid. The maximum pectin enzyme activity exhibited by the recombinant protein was 1200 U/mg. The exo-pelA gene of consisted of 1236 nucleotide sequences, which encoded a mature protein of 425 amino acids residues, in relation to the predicted molecular weight of ~48.7 kDa by MALDI-TOF-MS. To our knowledge, this is the first report on *A. guangheii* as a gut bacterium of freshwater fish, *Systomus sarana* that can be used for pectinase enzyme production. Significantly the present study, after *exo-pelA* gene expression the bacterial strain was enzyme production yield is very high. So, we can further experiment with large-scale processes for pectinase enzyme production. I strongly recommended that pectinase enzyme will be used for industries, like food, pharmaceutical, and leather.

Declarations

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Author contributions All authors brainstormed and came up with this study concept. NT provided the chemicals, equipment for the related microbiology tests and Response Surface Methodology (RSM-software) and related guidelines. BV provided the cloning vector, expression strain and related guidelines. PA assisted in review and editing for the manuscript. SD assisted in Writing – review and editing, data curation of the manuscript. PP provided the full manuscript language correction, conceptualization, writing – review & editing – original, draft. AD provided the bacteria carried out all the experiment, compiled and analyzed the resultant data and compiled this paper with insights from all the other authors.

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Conflict of interest All authors declare that they have no conflict of interest.

Ethics approval and consent to participate According to the Committee for the Purpose of Control and Supervision of Experiments on Animals-CPCSEA (under Ministry of Environment, Forests and Climate Change, Government of India), the edible fish which are used for laboratory experiments are exempted from obtaining Institutional Animal Ethics Committee (IAEC) approval. The study animal that we have utilized for the present work is *Systomussarana*(Hamilton, 1882) which is an edible fish, and hence the necessity did not arise to acquire the approval from IAEC. However, we have followed the OECD Guidelines for safe handling of experimental animals.

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Tables

Table 1 a) Composition of various experiments of Box-Behnken for independent variables and response; **b)** ANOVA of response surface quadratic model for pectinase enzyme; **c)** Plackett-Burman design matrix for 11 variables and their levels along with predicted and experimental values of pectinase activity; **d)** Regression analysis of the Plackett-Burman design for pectinase production.

a

Run	Maltose	Yeast extract	pH	Temperature	Incubation time	Pectinase Enzyme		
						Experimental value	Predicted value	
1	0.50	2.25	6.50	45.00	32.00		298	304.94
2	2.25	2.25	6.50	27.50	32.00		369	369.12
3	4.00	2.25	6.50	27.50	4.00		308	303.73
4	2.25	2.25	10.00	27.50	60.00		309	312.52
5	4.00	2.25	10.00	27.50	32.00		284	294.83
6	2.25	2.25	3.00	27.50	4.00		321	310.65
7	2.25	2.25	10.00	45.00	32.00		300	301.10
8	2.25	4.00	6.50	27.50	4.00		291	302.06
9	2.25	0.50	6.50	45.00	32.00		328	317.77
10	4.00	2.25	6.50	45.00	32.00		303	294.94
11	0.50	4.00	6.50	27.50	32.00		320	311.75
12	4.00	2.25	3.00	27.50	32.00		300	296.83
13	0.50	0.50	6.50	27.50	32.00		310	308.25
14	2.25	0.50	10.00	27.50	32.00		328	322.67
15	4.00	4.00	6.50	27.50	32.00		297	298.25
16	2.25	2.25	6.50	10.00	60.00		327	325.50
17	4.00	0.50	6.50	27.50	32.00		300	307.75
18	0.50	2.25	3.00	27.50	32.00		304	302.83
19	4.00	2.25	6.50	27.50	60.00		289	295.10
20	2.25	0.50	6.50	27.50	60.00		316	307.94
21	2.25	2.25	6.50	27.50	32.00		372	369.12
22	2.25	2.25	10.00	10.00	32.00		321	312.73
23	2.25	4.00	6.50	10.00	32.00		315	322.40
24	2.25	0.50	6.50	10.00	32.00		309	314.40
25	2.25	2.25	6.50	27.50	32.00		365	369.12
26	2.25	4.00	3.00	27.50	32.00		315	320.67
27	2.25	2.25	6.50	27.50	32.00		369.8	369.12
28	2.25	4.00	6.50	27.50	60.00		331	322.44
29	2.25	2.25	10.00	27.50	4.00		314	299.65
30	2.25	4.00	10.00	27.50	32.00		297	296.67
31	2.25	2.25	6.50	27.50	32.00		368.9	369.12
32	0.50	2.25	10.00	27.50	32.00		290	302.83
33	2.25	2.25	6.50	27.50	32.00		370	369.12
34	0.50	2.25	6.50	27.50	4.00		311	299.23
35	4.00	2.25	6.50	10.00	32.00		316	305.56
36	2.25	2.25	6.50	45.00	4.00		304	315.00
37	2.25	0.50	3.00	27.50	32.00		300	300.67
38	0.50	2.25	6.50	10.00	32.00		305	309.56
39	2.25	2.25	3.00	45.00	32.00		301	306.10
40	2.25	4.00	6.50	45.00	32.00		312	303.77
41	2.25	2.25	6.50	10.00	4.00		297	304.13
42	2.25	2.25	3.00	27.50	60.00		296	303.52

43	2.25	0.50	6.50	27.50	4.00	311	322.56
44	2.25	2.25	6.50	45.00	60.00	297	299.38
45	2.25	2.25	3.00	10.00	32.00	314	309.73
46	0.50	2.25	6.50	27.50	60.00	315	313.60

Con.

b

Source	Sum of squares	DF	Mean square	*F* value	*p*-value Prob > F
Model	23264.33	20	1163.216	12.91004	< 0.0001*
A	196	1	196	2.17532	0.1527
B	36	1	36	0.399549	0.5331
C	4	1	4	0.044394	0.8348
D	232.5625	1	232.5625	2.581112	0.1207
E	33.0625	1	33.0625	0.366947	0.5501
AB	42.25	1	42.25	0.468915	0.4998
AC	1	1	1	0.011099	0.9169
AD	9	1	9	0.099887	0.7546
AE	132.25	1	132.25	1.467786	0.2370
BC	529	1	529	5.871146	0.0230
BD	121	1	121	1.342927	0.2575
BE	306.25	1	306.25	3.398938	0.0771
CD	16	1	16	0.177577	0.6771
CE	100	1	100	1.109857	0.3022
DE	342.25	1	342.25	3.798487	0.0626
A^2	12055.52	1	12055.52	133.799	< 0.0001*
B^2	6051.879	1	6051.879	67.16722	< 0.0001*
C^2	9794.182	1	9794.182	108.7015	< 0.0001*
D^2	7381.879	1	7381.879	81.92833	< 0.0001*
E^2	7810.97	1	7810.97	86.69062	< 0.0001*
Residual	2252.542	25	90.10167		
Lack of Fit	2252.542	20	112.6271	1.27650	0.39876
Pure Error	0	5	0		
Cor Total	25516.87	45			

A, Maltose; B, Yeast extract, C, pH; D, Temperature; E, Incubation time. *=Significant, SD=9.4928, Mean=315.73913, C.V.%=3.0063, PRESS=9010.16667 R^2=0.9117, Adjusted R^2=0.8411, Predicate R^2=0.6468, Adeq Precision=11.7200.

Con.

c

Source	Sum of Squares	df	Mean Square	*F* Value	*p*-value Prob > F
Model	21541.6667	10	2154.16667	54.88322718	0.0008*
A	2133.33333	1	2133.33333	54.35244161	0.0018*
B	5208.33333	1	5208.33333	132.6963907	0.0003*
C	133.333333	1	133.333333	3.397027601	0.1391
D	8.33333333	1	8.33333333	0.212314225	0.6689
E	3008.33333	1	3008.33333	76.64543524	0.0009*
F	3008.33333	1	3008.33333	76.64543524	0.0009*
G	300	1	300	7.643312102	0.0506*
H	1200	1	1200	30.57324841	0.0052*
J	6533.33333	1	6533.33333	166.4543524	0.0002*
L	8.33333333	1	8.33333333	0.212314225	0.6689
Curvature	28616.3333	1	28616.3333	729.0785563	<0.0001*
Residual	157	4	39.25		
Lack of Fit	0	1	0	0	1.0000
Pure Error	157	3	52.3333333		
Cor Total	50315	15			

A, Glucose; B, Maltose; C, Sucrose; D, Galactose; E, Lactose; F, Mannitol; G, Beef extract; H, Peptone; J, Yeast extract; K, $NaNO_3$; L, KH_2PO_4. *=Significant, SD=6.265, Mean=230.250, C.V. %=2.721, PRESS=279.111; R^2= 0.993; Adjusted R2=0.975; Predicate R^2= 0.994, Adeq Precision= 35.664.

Con.

d

Run	A: Glucose	B: Maltose	C: Sucrose	D: Galactose	E: Lactose	F: Mannitol	G: Beef extract	H: Peptone	J: Yeast extract	K: $NaNO_3$	L: KH_2PO_4	Pectinase enzyme (U/m	
												Predicated Value	Experime Value
1	1	5.5	0.5	3	5	2	4	3.5	4.5	2	1	243.83	225.67
2	1	1.5	0.5	3	1	6	4	0.2	4.5	6	5	245.33	245.54
3	5	5.5	4	0.1	1	2	4	0.2	4.5	6	1	233.44	215.32
4	3	3.5	2.25	1.55	3	4	2.25	1.85	3	4	3	315.23	309.40
5	3	3.5	2.25	1.55	3	4	2.25	1.85	3	4	3	305.45	295.07
6	5	1.5	4	3	1	6	4	3.5	1.5	2	1	205.73	200.10
7	1	1.5	0.5	0.1	1	2	0.5	0.2	1.5	2	1	255.30	240.37
8	1	5.5	4	3	1	2	0.5	3.5	1.5	6	5	230.71	225.55
9	3	3.5	2.25	1.55	3	4	2.25	1.85	3	4	3	315.54	310.68
10	5	5.5	0.5	0.1	1	6	0.5	3.5	4.5	2	5	210.67	205.75
11	1	5.5	4	0.1	5	6	4	0.2	1.5	2	5	145.88	130.80
12	5	1.5	4	3	5	2	0.5	0.2	4.5	2	5	245.20	235.58
13	1	1.5	4	0.1	5	6	0.5	3.5	4.5	6	1	253.59	250.64
14	3	3.5	2.25	1.55	3	4	2.25	1.85	3	4	3	310.90	300.69
15	5	1.5	0.5	0.1	5	2	4	3.5	1.5	6	5	200.45	190.72
16	5	5.5	0.5	3	5	6	0.5	0.2	1.5	6	1	110.95	110.45

Table 2 Corresponding to the 50 kDa protein peptide result.

Observed (m/z)	Mr (expt)	Mr (calc)	ppm	Peptide
914.5533	913.5460	913.5093	40.2	R.RIDNQLR.G
971.5170	970.5097	970.5308	-21.75	R.IDNQLRGR.S
1033.5737	1032.5664	1032.5240	41.1	R.EPLVEYQR.E
1107.5891	1106.5818	1106.5720	8.86	R.VDRNDVVYK.N
1187.6652	1186.6579	1186.6744	-13.86	-.MPSIIDRVLK.I + Oxidation (M)
1249.6373	1248.6300	1248.5986	25.1	K.FDAVVDDIAER.H
1284.6495	1283.6422	1283.6582	-12.45	K.AIQNAQAQVEGR.N
1372.6902	1371.6829	1371.6782	3.39	R.YNEGLHQAIEAK.E
1493.7820	1492.7748	1492.7409	22.7	R.ELVSDALLAYEDR.E
1523.8658	1522.8586	1522.8103	31.7	R.TPLIISGPAQGEANR.W
1529.8308	1528.8235	1528.7845	25.5	R.SILEGEDLQDRVR.R
1591.8530	1590.8457	1590.8253	12.8	R.EVIEAGGLYVVGTER.H
1791.7870	1790.7797	1790.8086	-16.15	R.HWQEHLYEMDYLK.E
1852.9938	1851.9865	1851.8673	64.4	R.MQATVDAVNLLEDDFR.A + Oxidation (M)
1996.9888	1995.9815	1996.0993	-59.03	R.TLFQAQISAAPAPTSLPGVK.D
2008.9975	2007.9902	2007.9684	10.9	K.RMQATVDAVNLLEDDFR.A + Oxidation (M)
2086.9128	2085.9055	2086.0443	-66.54	R.EVIEAGGLYVVGTERHDSR.R
2198.0626	2197.0553	2197.0435	5.38	R.IMNSPSIPDDMALEFGFVSK.A
2211.1359	2210.1287	2210.1590	-13.72	R.EAAIVAQAGRPGAVTVATNMAGR.G
2233.1165	2232.1092	2232.0303	35.4	R.EGYTMFQNMLAGIREDAVR.T + 2 Oxidation (M)
2384.0146	2383.0073	2383.2384	-96.96	K.DYVVLDGEVQIVDEHTGRVLK.G
2508.2178	2507.2105	2507.2768	-26.45	R.HADGESLDLLLPEAFAAVREAAGR.T
914.5533	913.5460	913.5093	40.2	R.EPLVEYQREGYTMFQNMLAGIR.E + Oxidation (M)
971.5170	970.5097	970.5308	-21.75	K.NHAREAAIVAQAGRPGAVTVATNMAGR.G + Oxidation (M)

Table 3 Statistics of the Ramachandran plot of pectinase enzyme.

Ramachandran plot	Pectinase enzyme	Percentage
Residues in most favored regions	313	94.0%
Residues in additional allowed regions	20	6.0%
Residues in generously allowed regions	0	0.0%
Residues in disallowed regions	0	0.0%
Number of non-glycine and non-proline residues	333	100.0%
Number of end-residues (excl. Gly and Pro)	2	
Number of glycine residues (shown as triangles)	36	
Number of proline residues	15	
Total number of residues	386	

Table 4 Effect of different metal ions on pectate lyase activity.

Metal Ions	Enzyme Activity (U/mg)
$FeCl_2$	1324.0 ± 38.7[a]
$CoCl_2$	1558.5 ± 9.3[a]
$CaCl_2$	1754.6 ± 20.0 [a]
$MgCl_2$	1839.3 ± 31.8[a]
$MnCl_2$	1628.2 ± 15.1[a]
Control	1252.4 ± 25.5[a]

(i) *Values are mean ± S.D. of 3 replicates; (a) values followed by different superscripts are significantly different at $P < 0.05$; (b) values followed by same superscripts are not significantly different at ($P < 0.05$).

Figures

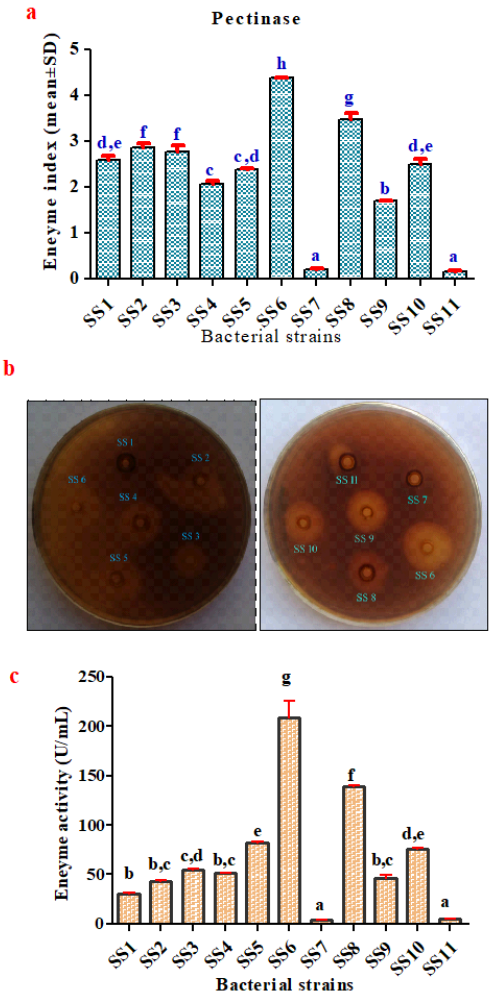

Figure 1

Pectinase enzyme producing bacterial strain- Enzyme index (a); Plate assay (b); Quantitative screening of pectinase enzyme producing isolated bacteria strains (c).

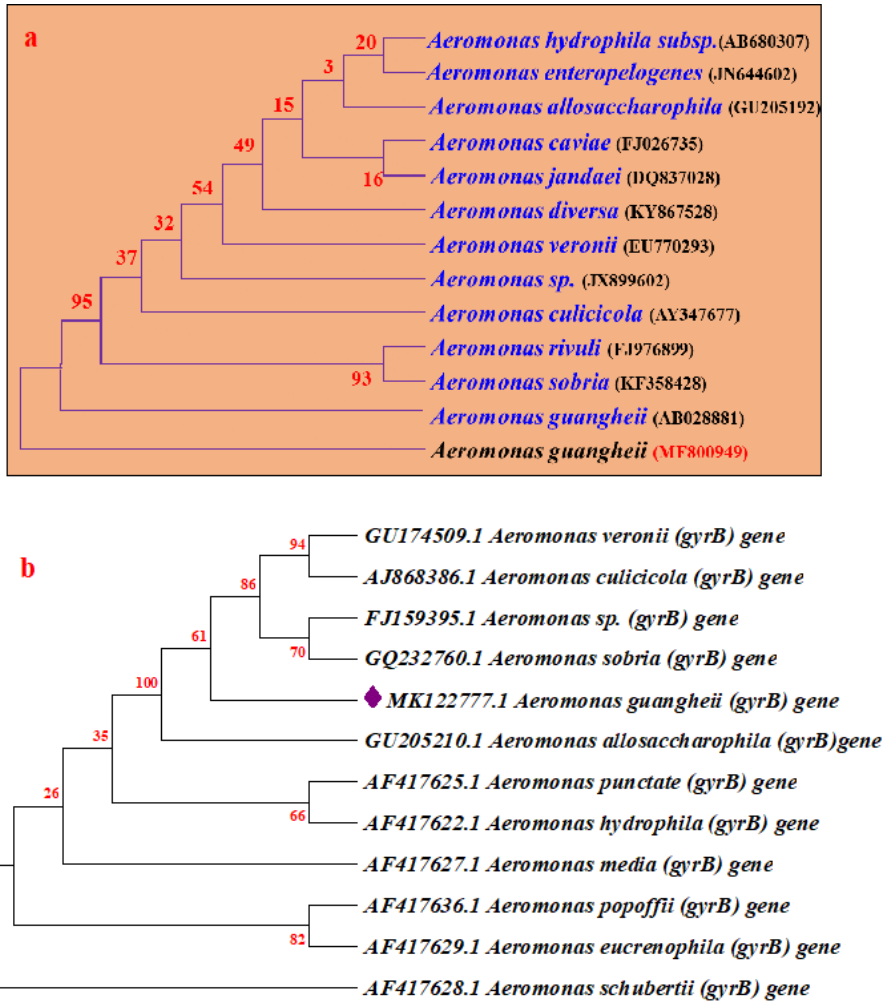

Figure 2

Neighbor-joining method for phylogenetic tree construction, based on the 16S rDNA sequence analysis, showing the relationships between the *Aeromonas guangheii* and related species- Genotypic (a); Phenotypic (b).

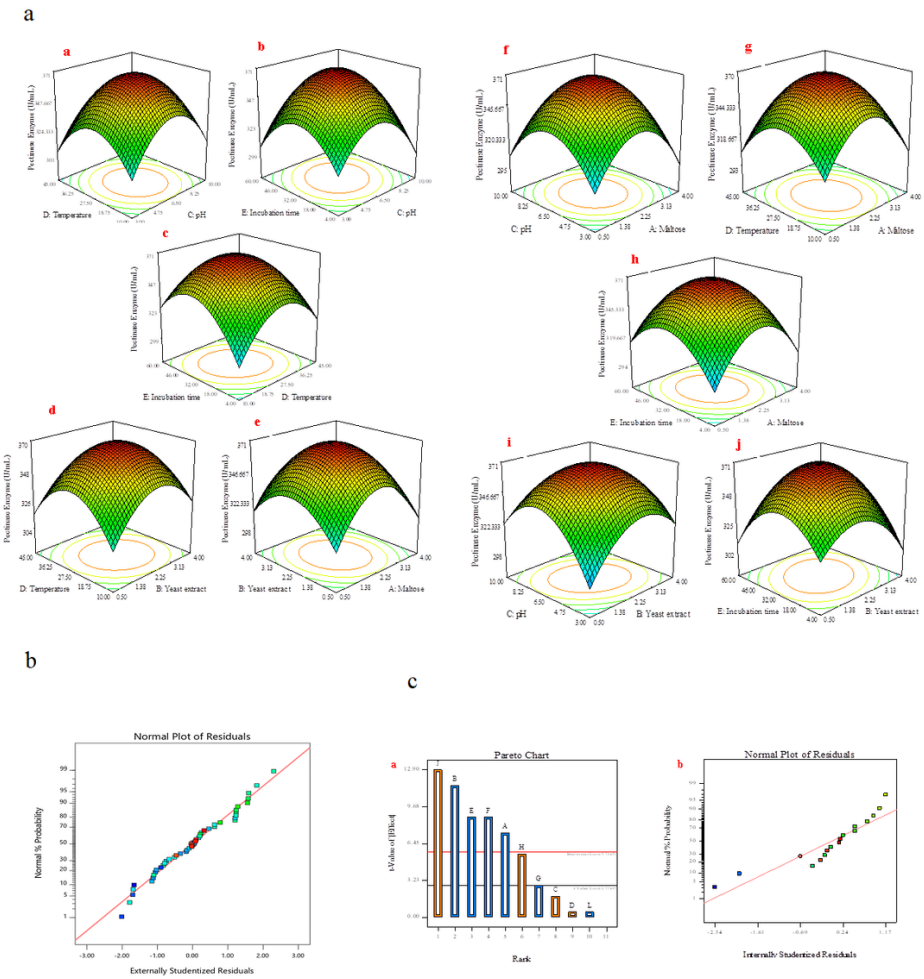

Figure 3

A) Response surface for extra-cellular pectinase (U/mg) production from *Aeromonas guangheii* in batch fermentation as an interaction between five parameters (a); Normal probability plot of studentized residuals (b). **B)** Pareto chart showing the most significant variables on *Aeromonas guangheii* pectinase production (a); Normal plot of residuals (b).

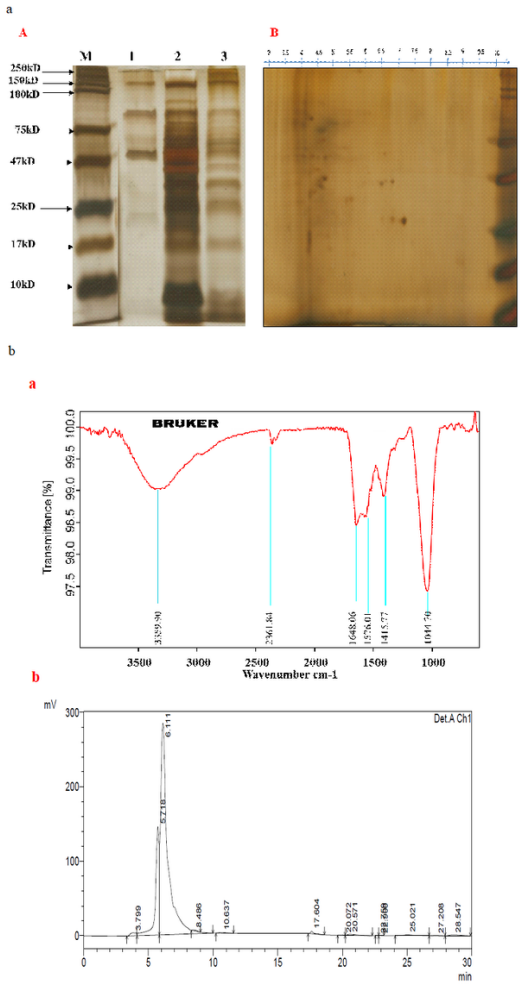

Figure 4

A) SDS-PAGE analysis of protein by the *Aeromonas guangheii*. M: Protein marker, (1) Purified protein, (2) Pectin substrate crude protein, (3) Culture crude protein (a). Two-dimensional gel electrophoreses proteins in *Aeromonas guangheii* (b). **B)** FTIR analyses of extract recombinant proteins (a); HPLC analyses of extract recombinant proteins (exo-type enzyme) (b).

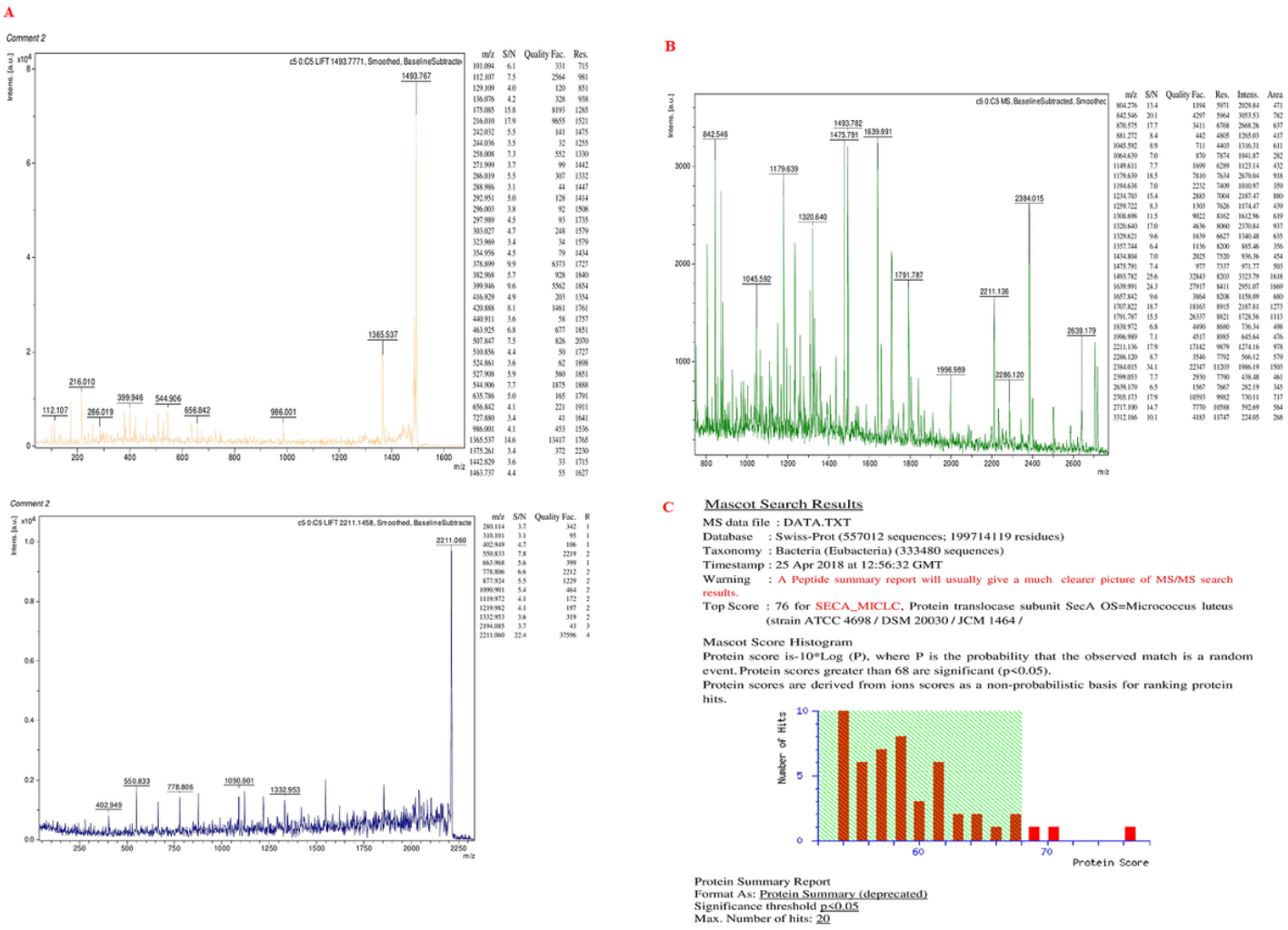

Figure 5

MALDI-TOF analysis: Intact mass determination (a); peptide spectrum (b); and Mascot score Histogram of band corresponding to 50 kDa (c).

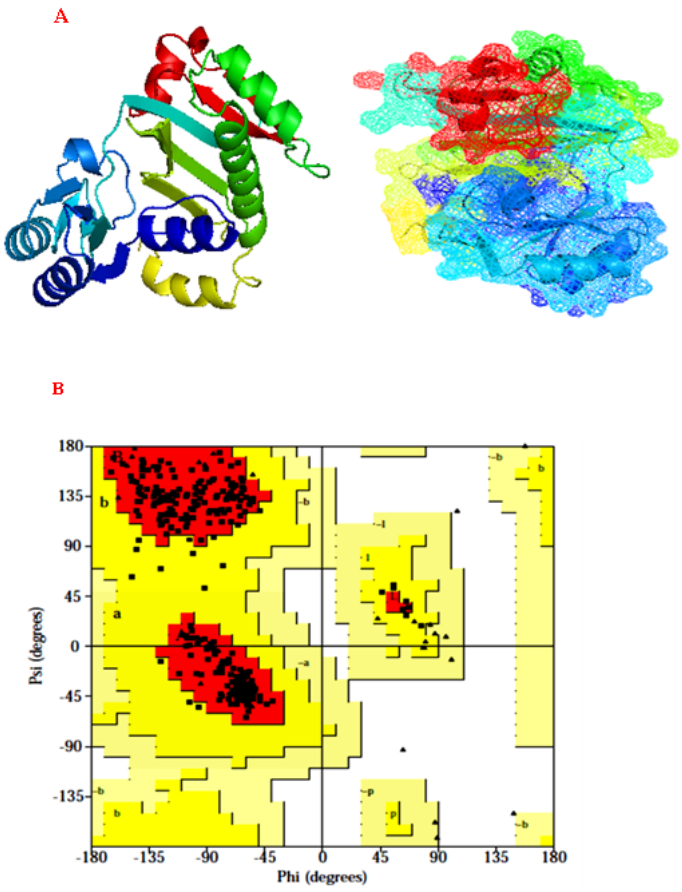

Figure 6

Homology modeling of purified pectinase of *Aeromonas guangheii* (SS6), Riben and Net diagram (a); Ramachandran plot (b).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementary.doc