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Production optimisation and characterisation of extracellular protease secreted by newly isolated *Bacillus subtilis* AU-2 strain obtained from *Tribolium castaneum* gut

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ABSTRACT

A novel protease secreting bacterial strain was isolated from the gut of *Tribolium castaneum* residing in stored *Glycine* max (soybean) seeds. The strain was identified as *Bacillus subtilis* AU-2. A response surface approach using Plakett-Burman Design (PBD) was used for the screening and a Central Composite Design (CCD) was used for the optimization of production parameters. An elevated protease production (580 U/mL) was achieved in optimised medium comprised of (g/L) soy bean meal, 10 g; glucose, 7.5 g; KH_2PO_4, 1.0 g; $CaCl_2$, 1.0 g and pH, 7.0 by using 3.5% inoculum at 37 temperature in agitated (60 rpm) batch culture after 48 h.

The three step enzyme purification achieved 26.81 fold purified protease which yielded a specific activity of 22773 U/mg and 34% recovery. The purified extracellular protease of *B. subtilis* AU-2 has molecular mass 38 kDa. The protease was documented as metallo-protease having optimum pH 7.5 and temperature 40. The purified protease showed appreciable endurance in presence of sodium dodecyl sulphate, Triton X-100, hydrogen peroxide and sodium per-borate. The protease efficiently digests proteinaceous substrates like casein, soybean flour, bovine serum albumin, haemoglobin, and egg albumin. The protease of newly isolated *B. subtilis* is now available for various food applications like soybean hydrolysate preparation, meat tenderisation, casein lysate preparation, milk clotting, and food waste treatment.

1. Introduction

Microbial proteases are prevalent enzyme for biotechnology sector, comprise of 60% utility and are largest selling biocatalyst for industrial applications (Li et al., 2013; Mokashe et al., 2018). The scientific understanding of enzymology has promoted the utility of this age-old food commodity in the current context of food applications. The enormous biodiversity of microorganisms advocates the search for new proteases and continuous innovation in existing knowledge of protease enzymology. Bacterial proteases exists in massive range of variants and specificity (Mokashe et al., 2018) and have been appreciated for their utilisation profile in food sectors like meat tenderisation, milk clotting, protein hydrolysate preparation, bioactive peptide synthesis, and allied food manufacturing process (Contesini et al., 2018). The safety of the enzyme secreting strain should be the principal attention in proposing usefulness of enzyme for food application (Pariza and Johnson, 2001).

The widening applications of proteases are dependent on bioprospecting for efficient protease secreting source. The insects are the most diverse and adapted to varied ecological niches; they harbours ~10 times more microbes in their gut than total cells of the insect (Rajagopal, 2009). The insect gut possesses enormously diverse microbiota which consists commensal, parasite and mutualistic microbe (Mrazek et al., 2008). The studies on insect-microbes symbiosis revealed that the insect do not possess the several enzymes to digests the nutrients and they depend on their gut microbiota for utilization of polymeric nutrients (Douglas, 2013). The dependence of several insects on microbes for lignocellulose utilization is well documented (Ni and Tokuda, 2013). Also, the gut microbiota has improved digestion efficacy in several insects (Santo Domingo et al., 1998; Broderick et al., 2004).

The present study is based on the assumption that the legume pod borer insect might harbour the proteolytic commensal bacteria which could secrete the efficient protease of industrial use. The legume seeds (*family* Fabaceae) consist of abundance of nutritious proteins (~40%)

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(Baudoin and Maquet, 1999). Majority of insects are reported to reside and live by causing damage to seeds in storage period. The proteinaceous seed kernel consumed by insects residing in seed metabolizes it to easily absorbable fundamental units by taking advantage of gut microflora (Priya et al., 2012). This study revealed the presence of novel protease secreting bacterium in the gut of insect *Tribolium castaneum* collected from stored soybean seeds. The aim of the research work is statistical production optimisation and characterisation of extracellular protease secreted by newly isolated *Bacillus subtilis* AU-2. The protease of newly isolated bacterial strain is suggested for commercial food applications viz. (i) soybean hydrolysate preparation, (ii) meat tenderisation, (iii) casein lysate preparation, (iv) milk clotting, and (v) food waste treatment.

2. Materials and methods

2.1. Chemicals and media

All chemicals and media were purchased from HiMedia, SD Fine (India), Sigma-Aldrich (USA).

2.2. Insect collection

Live insects were collected from six month naturally stored *Glycine* max seeds (Nandurbar, India). The collected insects were released in glass jar with a one inch layer of dried plaster of Paris at bottom. The containers were placed in individual plastic bags with sterile moist cotton to provide necessary humidity for transportation to the laboratory. The insects were sterilized by exposing to UV light for 1 min, to reduce external bacterial contamination.

Fifteen live insects randomly collected were rinsed with sterile distilled water 3 times. Then each insect was rinsed in 70% ethanol for 2 min, followed by three quick rinses of sterile phosphate-buffered saline (PBS) having composition, NaCl, 0.88 g; KCl, 0.02 g; Na_2HPO_4, 0.144 g; KH_2PO_4, 0.024 g in 100 mL distilled H_2O; pH 7.0 (1 ×). The insect were ice-anesthetized and then peripheral, anterior, and posterior parts were detached with sterile forceps, and the gut fluid was obtained in sterile PBS (1 ×).

2.3. Identification of insect

The morphological features of insect were documented and used for identification. Also, a phylogenetic trait was analysed by studying mitochondrial cytochrome oxidase gene of insect. The phylogenetic study was determined at National Centre for Microbial Resource (NCMR), Pune.

For this, adult insect collected was ground into fine powder by using a mortar and pestle. The total DNA was extracted from the insect by using the protocol of DNase easy Blood & Tissue Kit (Qiagen, Hilden, Germany). The quality of the extracted DNA samples were patterned on 0.8% agarose gel, and DNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (Nano Drop technologies, Willing Minton, USA). The extracted DNA samples were stored at −20°Cuntil further processing. The cytochrome oxidase (COI) gene sequence was amplified using primers, (COIf: 5′- GGTCAACAAATCAT AAAGATATTGG -3′ and COIr: 5′-TAAACTTCAGGGTGACCAAAAAA TCA-3′) (Folmer et al., 1994). The amplified products were directly sequenced using the ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing kit on a 3730xl Genetic Analyser (Applied BioSystems). The sequence data obtained was assembled and analysed using DNA sequence assembling software ChromasPro. The similarity search of newly generated COI gene sequences was performed on the NCBI database.

The COI sequences were aligned using Clustal X version 2.0 (Larkin et al., 2007), and the same sequences were grouped under one haplotype. The phylogenetic analyses based on the neighbor-joining (NJ) and maximum likelihood (ML) methods were performed using MEGA 5.05 (Tamura et al., 2011). The sequence was published on GenBank by assigning the accession number.

2.4. Isolation of bacteria from insect gut

The insect gut homogenate was quickly inoculated in Erlenmeyer flask containing enrichment medium - soybean meal broth comprising of (g/L) - soybean meal powder, 10; yeast extract, 1.0; and glucose, 1.0. The inoculated medium was incubated at 37°Cfor 72 h incubation on rotary shaker (100 rpm). After 72 h incubation, 0.1 mL aliquot of culture broth was spread on sterile skim milk agar medium containing (g/L)- skim milk powder, 28; yeast extract, 2.5; glucose, 1.0; agar, 30 and incubated at 37°C for 48 h. Among various isolated bacterial strains, the AU-2 strain exhibiting zone of proteolysis was selected for further identification.

2.4.1. Identification of bacterial strain

Various morphological, cultural, biochemical and ribotyping characteristics were examined for identification of isolated strain. The phylogenetic taxonomic depiction was determined on the basis of nucleotide sequences at National Centre for Microbial Resource (NCMR), National Centre for Cell Science, Pune. For the phylogenetic identification, the total genomic DNA of bacterial strain AU-2 was obtained as per Sambrook et al. (1989). Subsequently, the 16S rRNA gene was amplified from the total chromosomal DNA using universal eubacteria specific primer 16 F27 (5′-CCA GAGTTTGATCMTGGCTCA-3′) and 16 R1525 × P (5′TTC TGC AGTCTAGAAGGAGGTGWTCCAGCC-3′) by PCR. The reaction mixture (25 µL) consists of 10 × buffer (2.5 µL), 2 mM dNTP (2.5 µL), 10 pMol/l 16F27 (1.25 µL), 10 pMol 17R1525XP (1.25 µL), 10U Taq DNA polymerase (0.2 µL), template DNA (2 µL) and water (15.3 µL). The PCR amplification were attuned by setting the cycles - (i) denaturation at 95°C for 1 min, (ii) annealing at 55°C for 1 min, (iii) extension of annealing at 55°Cfor 1 min and (iv) final extension at 72°C for 10 min. The PCR was operated for 35 cycles on PCR cycler (Applied Biosystem PCR system). The amplified DNA was further purified with PEG-NaCl and incubated for 10 min at 37°C. The precipitate was collected by centrifugation at 16000 × *g* for 15 min at 4°C. The pellet was washed twice with 70% ethanol, dried under vacuum, re-suspended in distilled water at concentration of > 0.1 pmol/µL and the purified DNA was sequenced using BIGDYE terminator kit 3.1 CABI PerkinElmer, USA. The sequencing reactions were run on ABI-PRISM 31D automated sequencer (Model 3730; Applied Biosystem, USA).

The 16S rRNA nucleotide sequence obtained was aligned by BLAST analysis at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). The nucleotide sequence of AU-2 strain has been submitted to the GenBank database and assigned with accession number. For establishing phylogenetic relationship with other bacteria, currently accessible sequences at NCBI were used and performed multiple sequence alignment by using BLASTN 2.2.20 + .

2.5. Protease assay

The protease activity was assessed by spectrophotometric method described by Nakanishi et al. (1974). For this, 1.0 mL suitably diluted protease was allowed to react with 1.0 mL of buffered substrate. The buffered substrate consists of 0.65%, w/v casein prepared in 0.2 M Phosphate buffer having pH 7.5. The reaction mixture was kept at 40 °C. After 10.0 min the catalytic reaction was stopped by 3.0 mL chilled Trichloro-acetic acid (TCA) reagent. The released fragmented peptides were spectrophotometrically (UV-VIS 1700, Shimadzu, Japan) measured at 275 nm by using tyrosine as the standard. At pH 7.5 and temperature 40°C, the amount of protease required to produce the peptide-fragment equivalent to 1.0 µg tyrosine per min per mL is considered as one unit (U) of protease.

2.6. Growth of bacteria

The bacterial growth was determined in terms of cell density. For this a biomass was harvested by centrifugation (12000 × *g* for 10 min at 4°C) and washed thrice with cold distilled water. The washed bacterial biomass was vacuum dried at room temperature till a constant weight was attained.

2.7. Optimization physical and chemical parameter for protease production

Based on literature survey several provisional experiments were conducted based on one variable at a time (OVAT) approach to resolve the range of crucial growth factors like pH, temperature, agitation and inoculum. Also, the suitable carbon source and nitrogen source for protease production were screened. For this a basal medium consists of (g/L) KH_2PO_4, 1 g; $CaCl_2$, 1 g; $MgSO_4{\cdot}7H_2O$, 0.05 g; pH, 7.0 was supplemented with 1% w/v carbon source (starch, glucose, fructose, maltose, xylose, sucrose and molasses) individually. A control set without any carbon source was also kept. Also, various nitrogen sources (beef extract, bovine serum albumin, casein hydrolysate, casein, gelatin, peptone, skim milk, soy meal, and yeast extract)were supplemented (1%, w/v) individually in basal medium consists of (g/L) glucose, 1; KH_2PO_4, 1 g; $CaCl_2$, 1 g; $MgSO_4{\cdot}7H_2O$, 0.05 g; pH, 7.0. All inoculated flasks were incubated at 37°Cunder agitation (100 rpm) and the protease yield after 48 h.

2.8. Statistical optimization of protease production media

To evaluate the optimum parameter for maximum growth and protease production a software-aided statistical approach was used. The Plackett–Burman design (PBD) was employed for preliminary screening of factors. The screened and potentially influencing factors were further optimised by response surface methodology using Central Composite Design (CCD) for determining the optimum values of individual factors.

2.8.1. Plackett–Burman design (PBD)

Based on these trial experiment seven factors *viz*. pH, agitation, glucose, soybean meal, KH_2PO_4, $CaCl_2$ and inoculum subjected for screening for their influence on growth and protease production. A two level (low and high) of seven factors was tested by this approach viz. pH (6, 8); agitation (0, 120 rpm); glucose (0, 1.5 g/L); soybean meal (0.5, 1.5 g/L); KH_2PO_4 (0.1, 0.5 g/L); $CaCl_2$ (0.1, 0.5 g/L) and inoculum (1, 6 mL). Total twelve experiments designed by the Plackett-Burman method were performed in single block as described in Table 1. The protease activity and growth (responses) were evaluated and documented. These responses were analysed for getting regression equations.

2.8.2. Central composite design (CCD)

The five factors pH (6.0–8.0), agitation (0–120 rpm), glucose (0–1.5 g/L), soybean meal (0 to 5 g/L), and inoculum (1–6 ml) exhibited their significant influence on protease production were further subjected to a 2^n factorial Central Composite Design (CCD) for optimizing their values. The CCD suggested a set of 32 experiments which were accordingly performed (Table 2). Growth and protease production were documented as response. The response data were investigated by the Minitab software, the 3D contour plots generated to analyse the interaction among screened factors. Validity of chosen quadratic model predicted by Minitab software was confirmed experimentally.

2.9. Purification of protease

The *B. subtilis* strain AU-2 was cultured in optimised medium, the cell broth was centrifuged (12000 × *g*, 10 min, 4 °C). The cell-free supernatant was sequentially precipitated with increasing concentration of ammonium sulphate. The protein-precipitate recovered using ammonium sulphate (75% w/v) was dialyzed (dialysis membrane number 110, HiMedia, Mumbai) using phosphate buffer (pH 7.5). The proteins were further fractionated by using Sephadex-G-75 (Sigma, USA) and DEAE- cellulose (HiMedia, Mumbai, India) as described previously (Patil et al., 2016). All purification steps were executed at 4°C. The molecular weight and homogeneity of purified protease were evaluated with sodium dodecyl sulphate polyacrylamide gel electrophoresis (12.0%, SDS-PAGE) as per protocol of Laemmli (1970), the protein bands were visualised by Coomassie brilliant blue R-250 staining. The molecular mass of the protease was determined by comparing its electrophoretic mobility with commercial protein markers (Standard medium range protein marker, Genei, Bangalore, India).

2.10. Effect of temperature and pH on protease activity and stability

The effect of temperature on activity and stability of purified protease of *B. subtilis* AU-2 was evaluated at different temperatures (20–70 °C). Also, the thermal stability of enzyme was evaluated by incubating the protease at various temperature (25 to 75°C) for 30 and 60 min. The protease assay was performed at 40°C. Similarly, the effect of pH on purified protease on activity and stability was assessed over a wide pH range (6.0–10.0) using various buffers at 40°C. An aliquot of the purified protease of *B. subtilis* AU-2 and substrate was prepared with various buffers *viz*.- 0.2 M glycine-HCl buffer (pH 2.0); 0.2 M acetate–sodium acetate buffer (pH 3.0–4.0); 0.2 M monobasic–dibasic sodium phosphate buffer (pH 6.0–8.0); and 0.2 M carbonate–bicarbonate buffer (pH 9.0–10.0). For evaluating the stability at various pH, the purified protease aliquot were prepared in various buffers (as described above); for an hour and protease assay was performed at 40°C with

Table 1
Plackett-Burman (2-level factorial) experimental design for screening of various parameters; growth and protease yield of respective experiments using *Bacillus subtilis* AU-2.

Expt. No.	Glucose (%, w/v)	pH	KH_2PO_4 (%,w/v)	Soybean meal (%, w/v)	Agitation (rpm)	$CaCl_2$ (%, w/v)	Inoculum (%, w/v)	Protease activity (U/mL)	Growth Dry weight (mg/mL)
1	1.5	6	0.5	1.5	0	0.5	1	5.42 ± 0.69	0.09 ± 0.01
2	0.0	8	0.1	0.5	0	0.5	6	34.94 ± 1.30	0.32 ± 0.07
3	0.0	6	0.1	0.5	0	0.1	1	8.43 ± 1.78	0.1 ± 0.05
4	1.5	8	0.5	0.5	120	0.5	1	198.19 ± 7.62	1.12 ± 0.10
5	1.5	6	0.5	0.5	0	0.1	6	79.52 ± 4.12	0.61 ± 0.05
6	1.5	6	0.1	0.5	120	0.5	6	13.86 ± 2.01	0.71 ± 0.10
7	0.0	6	0.5	1.5	120	0.1	6	27.71 ± 3.59	0.38 ± 0.10
8	0.0	8	0.5	0.5	120	0.1	1	42.77 ± 1.90	0.41 ± 0.04
9	1.5	8	0.1	1.5	0	0.1	1	450.00 ± 6.12	2.04 ± 0.07
10	1.5	8	0.1	1.5	120	0.1	6	505.42 ± 5.52	2.18 ± 0.14
11	0.0	6	0.1	1.5	120	0.5	1	6.02 ± 0.98	0.1 ± 0.03
12	0.0	8	0.5	1.5	0	0.5	6	182.53 ± 4.52	1.22 ± 0.16

Table 2
Central composite design and respective responses (growth and protease yield) using *Bacillus subtilis* AU-2.

Expt. No.	Glucose (%,w/v)	pH	Soybean meal (%,w/v)	Agitation (rpm)	Inoculum (%,w/v)	Protease activity (U/mL)	Growth (Dry weight) (mg/mL)
1	0.75	7	0	60	3.5	274.10 ± 6.30	1.15 ± 0.05
2	1.5	8	0.5	120	1	340.96 ± 2.50	1.15 ± 0.02
3	1.5	6	1.5	120	1	162.65 ± 1.80	0.34 ± 0.04
4	0	8	0.5	120	6	314.46 ± 1.40	1.03 ± 0.03
5	1.5	8	1.5	0	1	460.84 ± 3.60	1.35 ± 0.05
6	0.75	9	1	60	3.5	151.20 ± 1.20	0.41 ± 0.04
7	0.75	7	1	−60	3.5	97.59 ± 1.40	0.94 ± 0.05
8	0.75	7	1	60	3.5	491.57 ± 4.50	1.23 ± 0.03
9	1.5	6	1.5	0	6	203.01 ± 2.30	1.43 ± 0.02
10	0	6	1.5	120	6	10.84 ± 1.10	0.14 ± 0.01
11	0.75	7	2	60	3.5	550.00 ± 4.80	1.09 ± 0.01
12	0.75	7	1	60	3.5	492.77 ± 4.50	1.23 ± 0.02
13	1.5	6	0.5	120	6	392.17 ± 3.50	0.84 ± 0.03
14	0	8	0.5	0	1	153.01 ± 2.20	0.61 ± 0.04
15	0.75	7	1	60	3.5	498.80 ± 3.40	1.24 ± 0.04
16	0.75	7	1	60	−1.5	0 ± 0	0 ± 0
17	1.5	6	0.5	0	1	257.83 ± 2.11	1.11 ± 0.01
18	0	8	1.5	120	1	334.94 ± 1.20	1.54 ± 0.02
19	0.75	7	1	60	3.5	501.20 ± 4.30	1.22 ± 0.04
20	0	6	0.5	120	1	5.42 ± 0.80	0.1 ± 0.00
21	0	6	0.5	0	6	3.01 ± 0.00	1.3 ± 0.01
22	0	6	1.5	0	1	5.42 ± 0.80	0.11 ± 0.02
23	2.25	7	1	60	3.5	124.10 ± 1.40	0.4 ± 0.03
24	−0.75	7	1	60	3.5	0 ± 0	0 ± 0
25	1.5	8	0.5	0	6	280.12 ± 2.40	0.7 ± 0.01
26	0	8	1.5	0	6	368.07 ± 1.10	1.3 ± 0.02
27	1.5	8	1.5	120	6	568.07 ± 1.20	2.3 ± 0.03
28	0.75	7	1	180	3.5	535.54 ± 2.30	1.21 ± 0.02
29	0.75	7	1	60	3.5	492.17 ± 3.50	1.29 ± 0.02
30	0.75	7	1	60	8.5	356.63 ± 3.40	1.5 ± 0.01
31	0.75	5	1	60	3.5	0 ± 0	0 ± 0
32	0.75	7	1	60	3.5	498.80 ± 4.50	1.25 ± 0.02

buffered casein substrate (pH-7.5).

2.11. Effect of inhibitors on protease activity

The interaction of protease with repertoire of well-known protease inhibitor like phenyl methyl sulfonyl fluoride (PMSF), *N*-ethylmaleimide, ethylenediaminetetraacetic acid (EDTA) and 1, 10 phenanthroline gives clue about type of protease. For this, the enzyme aliquots were prepared in buffer containing respective protease inhibitor (5 mM), and pre-incubated for 1 h at 27 °C. The enduring protease activities were assessed and equated with a control.

2.12. Effect of metal ions on protease activity

The effect of metal ions on protease activity was evaluated. In this experiment, purified protease was pre-incubated independently with 5mMchloride salt of respective metals ($BaCl_2$, $CaCl_2$, $CdCl_2$, $CuCl_2$, $FeCl_2$, $HgCl_2$, KCl, LiCl, $MgCl_2$, $MnCl_2$, NaCl, $NiCl_2$, $SnCl_2$, and $ZnCl_2$) for 1 h at 27°C. The influenced protease activity was assessed and comparing it with a control.

2.13. Effect of surfactants and oxidizing agents on protease

The activity of purified protease was also analysed in the presence of various surfactants, and oxidizing agents (1%, w/v). For this, the aliquot of purified enzyme was pre-incubated with anionic detergent (Sodium dodecyl sulphate), non-ionic detergents (Triton X-100, and Tween-20) and oxidizing agents (Hydrogen peroxide and sodium perborate) at 27°Cfor 1 h, and subsequently assayed for the residual protease activity.

2.14. Substrate utilization profile

The ability of protease to act against the range of natural substrates, like bovine serum albumin (BSA), casein, egg yolk albumin, gelatin, haemoglobin, and soybean flour was evaluated. For this, 1.0 mL of each substrate (2%), prepared in phosphate buffer (pH 7.5) was mixed with purified protease and incubated at 40°C for 10 min. One unit (U) of protease activity was defined as that amount of enzyme required to produce peptides equivalent to 1.0 µg tyrosine in the filtrate per minute per millilitre at pH 7.5 and 40 °C.

2.15. Statistical analysis

The reported enzyme units were the average value of results of three independent experiments. All the data are expressed as average ± standard deviation (SD). Statistical analysis was performed using Microsoft Excel. The production media and physical factors optimisation were achieved by response surface approach. A software package Minitab® 18.1 (Trial version) was used for the design of screening and optimisation experiments, data analysis, quadratic model building, response surface and 3-D contour plots generation to understand the interaction of different variables.

3. Results and discussion

3.1. Insect

The insect was collected from stored *Glycine* max seeds. It was identified as *Tribolium castaneum* (Coleoptera: Tenebrionidae) on the basis of the morphological features using the taxonomic key suggested by Bousquet (1990). The identification was further confirmed by analysing the phylogenetic attributes of insect. The partial DNA sequence of mitochondrial cytochrome oxidase (COI) gene of studied insect was

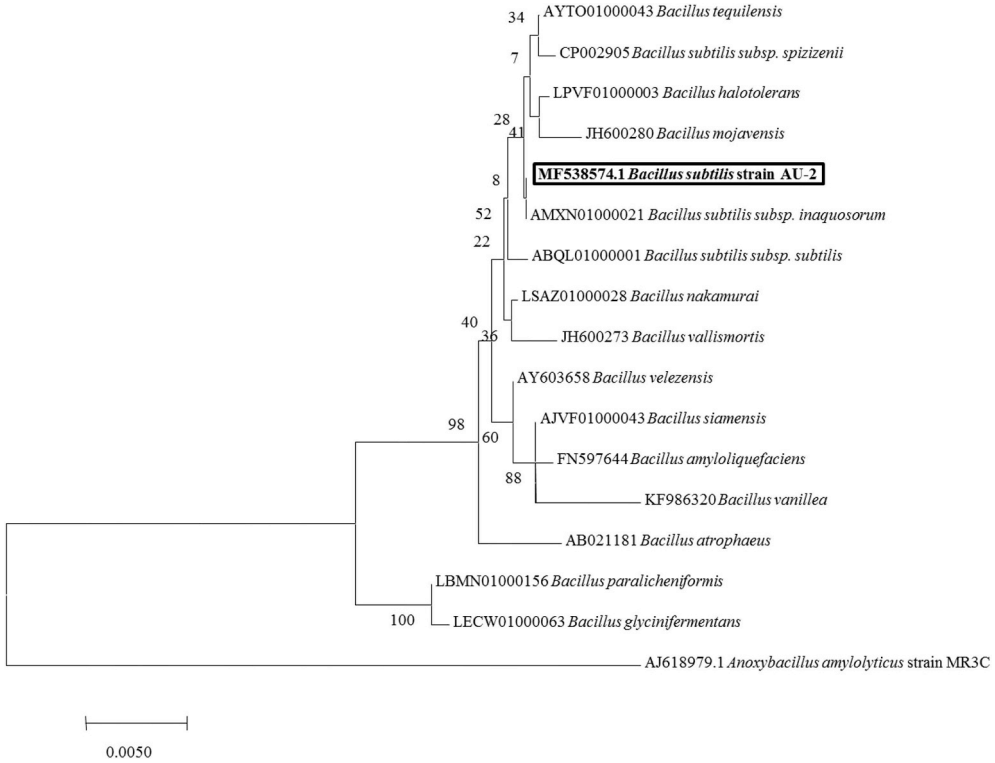

Fig. 1. Phylogenetic relationship of newly isolated strain *Bacillus subtilis* AU-2.

used for identification and the sequence had been submitted to GenBank databases with accession number: MH442992.

The *Tribolium castaneum,* also known as red flour beetle commonly is a tropical beetle pest of stored grains and pulses (Hagstrum and Subramanyam, 2009). The presence of *Tribolium castaneum* insect on stored soybean was also reported by LeCato and McCray (1973). These insects digestive system is adapted consume proteinaceous diet obtained from *Glycine* max seeds. Based on assumption that the gut microbiome of *Tribolium castaneum* (residing in *Glycine* max seeds)*;* could contain an efficient protease producing bacterial strain, further experimentation was lineated.

3.2. Microorganism

On basis of morphological, cultural, biochemical characteristics (Table S1) and phylogenetic analysis, the efficient protease secreting bacterial strain AU-2 was identified as *Bacillus subtilis.* The partial DNA sequence of 16S rRNA gene (1479 bp) of AU-2 strain was documented with accession number - MF538574.1 at NCBI database. This partial DNA sequence was also compared with related strains to establish the phylogenetic relationship with correlated bacterial strains (Fig. 1).

The present investigation has revealed the presence of protease secreting *Bacillus subtilis* in gut of *Tribolium castaneum*. The *Bacillus subtilis* is present as commensal in *Tribolium castaneum* gut was also documented previously (Kumari et al., 2011). The *B. subtilis* has been reviewed by the Food and Drug Administration (Centre for Veterinary Medicine) and included it in list of Direct-Fed microorganisms (US Food and Drug Administration, 1999). Also, *B. subtilis* was reported to be used for preparation of East Asian fermented foods –natto (Hosoi and Kiuchi, 2003). The Hong et al. (2009) documented that *B. subtilis* had adapted to survive as human gut commensals and mediate important roles like biofilm formation, and antimicrobial secretion. Likewise, the quorum sensing molecules (QSMs) secreted by *B. subtilis* strain JH 642 are found to be play a substantial role in safeguarding the intestinal health (Fujiya et al., 2007). The probiotic preparation supplemented with *Bacillus subtilis* are commercially available in several countries which includes - BioPlus-2B® (Denmark), Anaban™, and Biosporin® (Europe) (Elshaghabee et al., 2017). Moreover, *B. subtilis* strains have been conferred GRAS (Generally Recognized as Safe) status by the Food and Drug Administration (Contesini et al., 2018).

Hence, by considering the safe nature of this insect gut commensal strain, the protease obtained from it could offer potential application in food industry. The food manufacturers are inclined towards safe and mild enzymatic methods to replace traditionally used strong acid hydrolysis.

3.3. Statistical optimization of protease production media and physical factors

The suitability of any protease secreting strain for industrial application depends on enzyme yield. The protease production and growth is depends on the media composition and physical factors. In current investigation, total seven factors (glucose, pH, KH_2PO_4, soybean meal, agitation, $CaCl_2$, and inoculum size) were selected for primary screening by Plackett–Burman design (PBD). All the experimental sets were incubated for 48 h, as it yields highest enzyme and further prolonged incubation not contribute to increase protease yield. The experimental PBD design with respective responses (protease activity and growth) is summarised in Table 1. Based on the regression coefficient values of responses; Glucose (79.2); pH (106.0); Potassium dihydrogen phosphate (−40.2); Soybean meal (66.7); Agitation (2.8); Calcium chloride (−56.1); and Inoculum (11.1); further five factors which showed positive influence on protease production were further selected for central composite design (CCD) of response surface method. The K_2HPO_4 and $CaCl_2$ showed a minimum effect were taken in low-level in subsequent experimentation.

By using various levels of glucose, pH, soy bean meal, agitation, and inoculum; the CCD designed experiments were accomplished consequently (Table 2). The responses (growth and protease activity) were inputted to Minitab software to generate the quadratic regression equations and 3D contour plots.

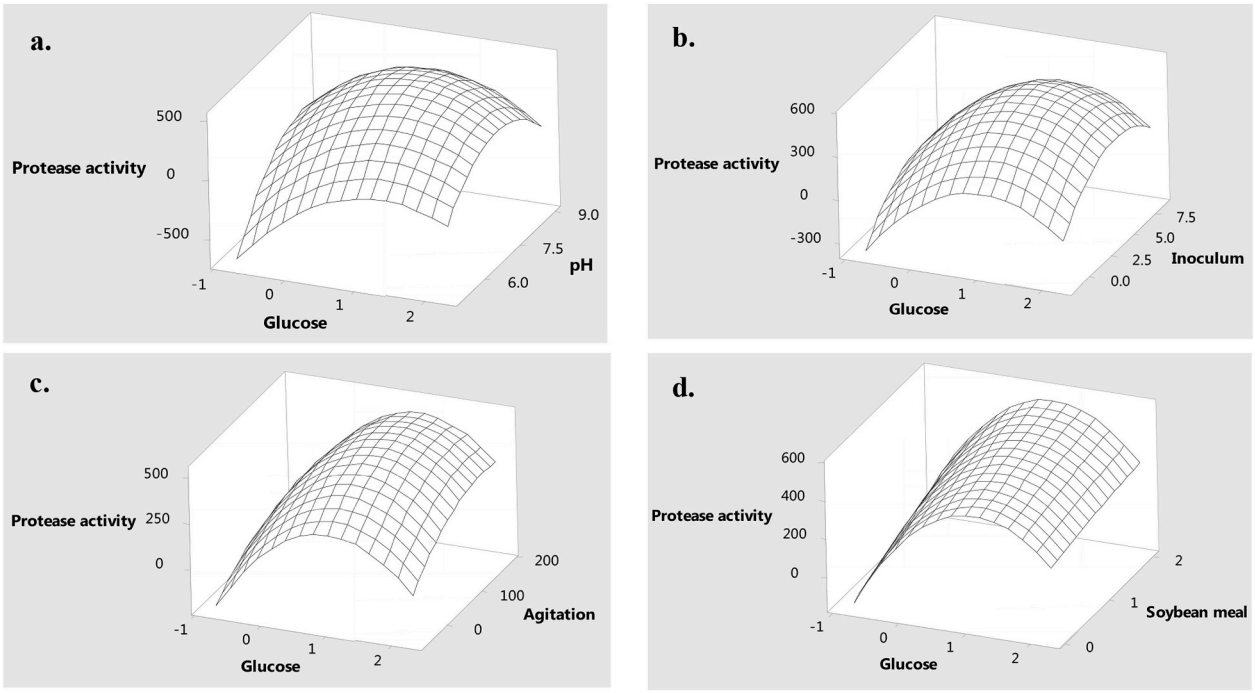

Fig. 2. The interacting prominent effects of optimised parameters **(a)** glucose and pH; **(b)** inoculum and glucose; **(c)** glucose and agitation; and **(d)** glucose and soy bean meal on protease production by *Bacillus subtilis* AU-2.

3.3.1. Quadratic regression equation

To get 3D contours graphs, the protease activity was analysed by taking two factors at a time and other three at a fixed level. Fig. 2 shows the various interacting effects of glucose, soybean meal, pH, agitation, and inoculum. The resulted protease production based on their mutual interaction is depicted as response surface plots, which shows the combined effects of these factors. Each 3D contours graph generated is almost convex contour plot, which designates that there were precise optimum levels.

Fig. 2a shows that increasing the concentration of glucose and pH leads to elevate protease production by *B. subtilis* AU-2, while further raise (i) beyond 1% glucose and, (ii) pH towards alkaline range has lowered the protease production. The *B. subtilis* AU-2 strain grew in pH range of 6.0 to 9.0; while the maximum protease production was detected at pH 7.0.

Also, the protease production was promoted with increase of agitation rate and inoculum level up to optimum frontier, while further boost of these factors has lowered the protease production by *B. subtilis* AU-2, as shown Fig. 2b and c. The protease production by altering the glucose concentration with specific bio-inoculum load and efficient utilization of available oxygen was assessed by this interaction.

As shown in Fig. 2d, the presence of organic nitrogen source – soybean alone was not adequate to maximize the protease production; addition of carbon source like glucose was essential for the improving the protease production of the strain. Although, the glucose promoted protease production up to 1% concentration further increase in glucose level has lowered the protease yield. Similarly, protease production was detected to be suppressed by glucose beyond 1.0% concentration in almost all interactions – glucose-pH, glucose-inoculum, and glucose-agitation (Fig. 2a, b and 2c); this emphasizes that excess glucose beyond the optimum concentration might repressed the protease production or altered the osmoregulation which further adversely affected the growth of strain and protease secretion. The soybean meal showed vis-à-vis response on protease production and even 2% soybean meal was endured by the strain. For large scale commercial enzyme production, the use of cost-effective media is vital. The ability to utilize soya bean by the *B. subtilis* AU-2 is a positive gain as the soybean meal is a comparatively cheap and easily obtainable media component, its industrial appropriateness for economical protease production at large scale is an encouraging factor.

The optimum values of selected factors for maximum protease production were calculated using the quadratic regression equation.

Protease activity = 4167 + 639 Glucose + 1192 pH + 65.5 Inoculum - 649 Soybean meal+ 0.19 Agitation - 159.2 Glucose*Glucose - 86.1 pH*pH- 9.68 Inoculum*Inoculum - 8.1 Soybean meal*Soybean meal- 0.00718 Agitation*Agitation - 42.6 Glucose*pH + 0.8 Glucose*Inoculum- 20.0 Glucose*Soybean meal + 0.175 Glucose*Agitation + 1.6 pH*Inoculum+ 115.0 pH*Soybean meal + 0.203 pH*Agitation - 2.4 Inoculum*Soybean meal+ 0.194 Inoculum*Agitation - 0.67 Soybean meal*Agitation

The optimized medium evaluated using response surface approach consists (g/L) - soy bean meal, 10 g; glucose, 7.5 g; KH_2PO_4, 1 g; $CaCl_2$, 1 g and the pH should be 7.0. The batch culture consists of the optimized medium inoculated with 3.5% inoculum and agitated at 60 rpm at 37°C yielded 580 U/mL after 48 h. Further incubation has not contributed to increase the protease yield and leads to slight decline in protease level. Hence the incubation period for optimized protease production was kept 48 h. Protease activity obtained from fermentation medium using OVAT approach was 158 U/mL. Hence, the systematic response surface approach has enhanced to 4.0 fold improvements as compare to the production using basal medium. Relatively higher fold (7.0-fold) enhancement in enzyme production was achieved using response surface approach from *Bacillus subtilis* FBL-1, which yielded 578 U/mL protease in optimized medium (Kim et al., 2016).

3.4. Purification of protease

Extensively purified protease has been a prerequisite to characterise the enzyme for studying the biochemical profile and to confirm the suitability of enzyme to use in food biotechnology. Various approaches have been utilised for purification of extracellular proteases secreted by *Bacillus* strains (Mokashe et al., 2018); in this study the first step of purification was concentration of proteins by ammonium sulphate

Table 3
Purification summary for protease obtained from *Bacillus subtilis* AU-2.

Purification steps	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification fold
Crud broth	309.8 ± 10.7	264257 ± 19514	853 ± 66	100 ± 0	1.00 ± 0.00
$(NH_4)_2SO_4$ precipitation (75%)	229.3 ± 15.8	208635 ± 13092	908 ± 7	79 ± 1	1.07 ± 0.09
Ion exchange-DEAE-Cellulose	16.9 ± 1.5	131225 ± 3931	7782 ± 623	50 ± 2	9.16 ± 1.06
Gel filtration-Sephadex G-75	3.8 ± 0.02	88675 ± 1870	22773 ± 361	34 ± 2	26.81 ± 2.47

precipitation method. Subsequently, chromatographic separation was carried by using DEAE-cellulose and Sephadex G-75 columns. The sequential three step purification scheme yielded 26.81-fold purification with a specific activity of 22773 U/mg and 34% recovery (Table 3). The purification results were consistent with the purification strategy employed previously for isolation of proteases from various strains of *Bacillus subtilis*. A 24% recovery and 27.63 fold purified protease with 11.33 U/mg specific activity (Maruthiah et al., 2013) and 17.87% recovery and 10 fold purified protease with 87.79 U/mg specific activity (Sathishkumar et al., 2015) were obtained from *Bacillus subtilis* AP-MSU 6 and *Bacillus subtilis* GA CAS8 respectively.

The purified protease of *B. subtilis* AU-2 was detected as a single band on polyacrylamide gel. The molecular mass of extracellular protease of *B. subtilis* AU-2 was estimated as 38 kDa by SDS-PAGE (Fig. 3). The documented results are in consistence with recently reported 37.6 kDa metallo-protease from *Bacillus subtilis* (Si et al., 2018). The molecular weight of the purified proteases of different strains of *Bacillus subtilis* was determined as 44 kDa (Yang et al., 2000) and 41 kDa (Sathishkumar et al., 2015). In contrary, several reports described low molecular-weight extracellular proteases (15–28 kDa) from different strains of *Bacillus subtilis* (Adinarayana et al., 2003; Kim and Kim, 2005; Maruthiah et al., 2013; Rehman et al., 2017).

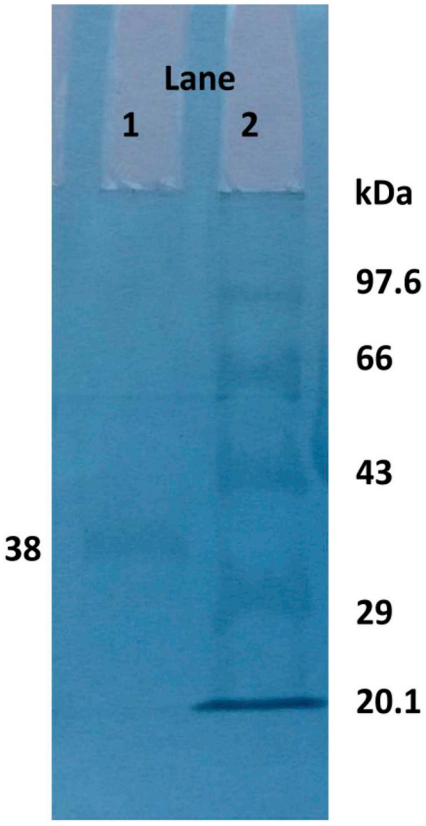

Fig. 3. Electrophoretic separation (SDS-PAGE) of purified protease of *Bacillus subtilis* AU-2; Lane 1: purified protease, and Lane 2: standard molecular weight markers.

The purified protease of *B. subtilis* AU-2 also confirmed to be homogenous by using gel electrophoresis was further subjected for biochemical characterization. The biochemical characterization of the protease is an authoritative obligation for newly reported enzyme as the applicability enzyme in food applications depends on various factors like sensitivity to pH, temperature, and ability to remain active and stable in presence of several surfactants, salts, and oxidising agents.

3.5. Effect of pH on protease activity and stability

Most of the proteases obtained from *Bacillus sp.* are alkaline proteases (Maruthiah et al., 2013; Si et al., 2018) with exception of protease of *Bacillus subtilis* KT004404 which has optimum pH 6.0 (Rehman et al., 2017). The protease of *B. subtilis* AU-2 studied in this investigation was active in the broad pH range of 6.0–10.0 with optimum activity at pH 7.5. It was remain active and stable at neutral pH while also showed appreciable stability in alkaline pH range compare to acidic pH (Fig. 4). Henceforth, the protease produced by *B. subtilis* AU-2 is a neutral protease like extracellular protease secreted by *B. subtilis* Y-108 (Yang et al., 2000).

Several food preparation are processed using a neutral protease hence protease from *B. subtilis* AU-2 could be utilized like commercially available Neutrase® in food industry. The ability of protease active at neutral to slightly alkaline pH range offers its potential to apply as key commodity for fish protein hydrolysate preparation.

3.6. Effect of temperature on protease activity and stability

Purified protease of *B. subtilis* AU-2 has showed activity in broad temperature range of 20–60°Cwith the optimum temperature 40 °C(Fig. 5a). The protease get totally inactivated at 70°C. Also, thermal endurance was determined after 30 and 60 min at various temperature 25–65°C (Fig. 5b); the enzyme has retained 74% and 62% activity after heating at 55°C for 30 and 60 min respectively. The enzyme get rapidly inactivated beyond 55°C. These results are in accordance with

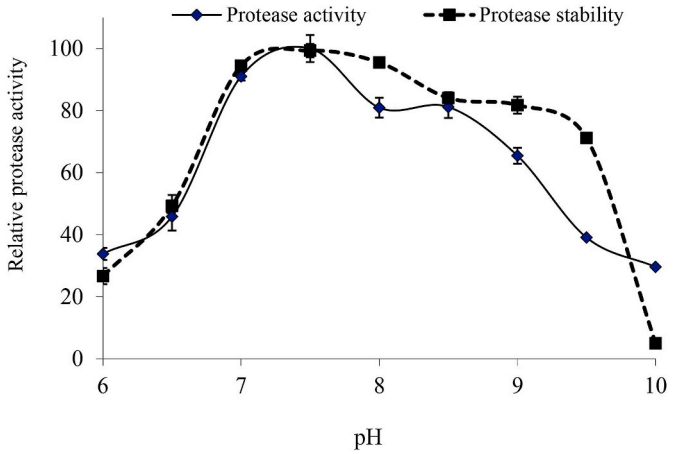

Fig. 4. Effect of pH on enzyme activity and stability of purified protease obtained from *Bacillus subtilis* AU-2.

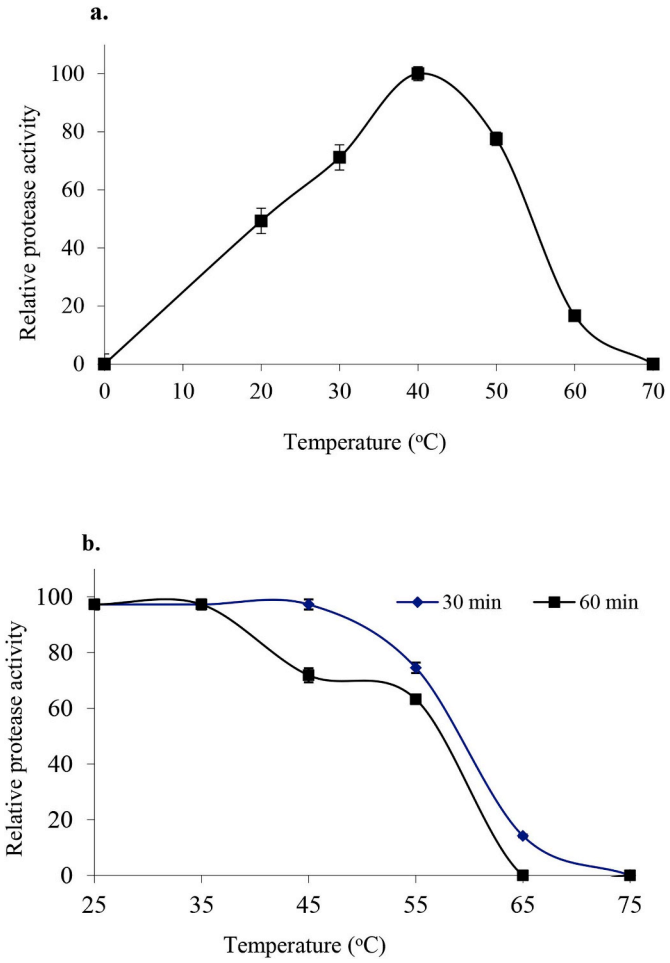

Fig. 5. (A) Effect of temperature on enzyme activity of purified protease obtained from *Bacillus subtilis* AU-2; **(B)** Thermal stability evaluated at various temperature after 30 and 60 min.

extracellular protease of *Bacillus subtilis* AP-MSU6 (Maruthiah et al., 2013). Previously, thermo-stable proteases having optimum temperature 50-55°Cwere reported from *Bacillus subtilis* Y-108 (Yang et al., 2000) and *Bacillus subtilis* KT004404 (Rehman et al., 2017).

3.7. Effect of inhibitors on protease

The effect of various inhibitors on protease activity is summarized in Table 4. The enzyme was inhibited by EDTA, and 1, 10 phenanthroline suggesting that the protease of *B. subtilis* AU-2 is the metalloprotease. Similarly, metallo-proteases were reported from *Bacillus subtilis* KT004404 (Rehman et al., 2017), *Bacillus subtilis* Y108 (Yang et al., 2000; Si et al., 2018). In addition, serine proteases form *Bacillus subtilis* PE-11 (Adinarayana et al., 2003) and *Bacillus subtilis* DR8806 (Farhadian et al., 2015) have also been described.

3.8. Effect of surfactant and oxidizing agents on protease

The effect of various chemicals such as surfactants, and oxidising agent on protease activity was examined. The protease showed appreciable endurance (80 to 96%) in presence of Tween-20, SDS, Triton X-100, H_2O_2 and sodium per-borate) as summarised in Table 4. Comparable outcomes were documented for metallo-protease obtained from *Bacillus subtilis* FBL-1 (Kim et al., 2016).

Food grade hydrogen peroxide (H_2O_2) used as a strong oxidising agent and effective antimicrobial agent in food industry. The hydrogen peroxide is used to improve or preserve the quality of margarine, cheese, instant tea, wine, or juice, corn syrup, and starch. These foods are treated with 0.05 to 1.5% (v/v) hydrogen peroxide. The capability to withstand active conformation in presence of H_2O_2 by protease of *B. subtilis* AU-2 could advises its utility in food industry.

Table 4
Effect of various activators/inhibitors on the purified protease of *Bacillus subtilis* AU-2.

Inhibitors/activators	Relative protease activity (Mean ± SD)
Protease inhibitors	
Control	100.36 ± 0.75
PMSF (5 mM)	98.77 ± 2.47
N-ethylmleimide (5 mM)	94.06 ± 4.49
1, 10 Phenanthroline (5 mM)	10.51 ± 1.36
EDTA (1 mM)	19.55 ± 1.50
EDTA (5 mM)	16.22 ± 1.03
Surfactant and oxidising agents (1%)	
Triton X-100	83.42 ± 1.21
Sodium dodecyl sulfate (SDS)	85.45 ± 3.62
Tween 20	77.55 ± 1.80
Hydrogen peroxide (H_2O_2)	93.63 ± 1.78
Sodium per-borate	96.81 ± 0.55
Metal ions (5 mM)	
$BaCl_2$	96.09 ± 3.60
$CaCl_2$	112.24 ± 1.95
$CdCl_2$	53.73 ± 6.65
$CuCl_2$	69.51 ± 1.52
$FeCl_2$	105.36 ± 2.30
$HgCl_2$	0.29 ± 0.33
KCl	107.82 ± 1.40
LiCl	95.66 ± 2.84
$MgCl_2$	110.35 ± 1.70
$MnCl_2$	117.02 ± 3.14
NaCl	91.46 ± 0.38
$NiCl_2$	69.73 ± 1.93
$SnCl_2$	90.95 ± 2.89
$ZnCl_2$	105.07 ± 1.33

3.9. Effect of metal ions on protease

The relative activity of protease was conserved in presence of Ba^{2+}, Li^+, Na^+, and K^+, (5 mM) salts. The protease activity was elevated in the presence of Ca^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} as compare to control; however the enzyme was significantly get inhibited by Sn^{2+} Cd^{2+}, Cu^{2+}, Hg^{2+}, and Ni^{2+}. The effect of metal ions on protease activity of *B. subtilis* AU-2 was shown in Table 4.

Similar effect of metal ions on protease activity has been testified earlier. Metal ions like Fe^{2+}, Hg^{2+},and Cu^{2+} have lowered the activity; while Co^{2+}, Ca^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} have enhanced the activity of protease obtained from *Bacillus subtilis* KT004404 (Rehman et al., 2017). Also, protease from *Bacillus subtilis* DR8806 showed enhanced activity in presence of metal ions like Ca^{2+}, K^+, Mg^{2+}, Fe^{2+} while the activity was lowered in the presence of Hg^{2+}, Ba^{2+}, and Cu^{2+} (Farhadian et al., 2015).

3.10. Substrate specificity of protease

The protease of *B. subtilis* AU-2 has ability to digest several proteinaceous substrates. The purified protease was tested with different natural proteinaceous substrates like bovine serum albumin, casein, egg albumin, gelatin, haemoglobin, and soybean flour. The purified enzyme has substrate utilisation profile as - casein (277 ± 12 U/mL); > soybean flour (250 ± 3 U/mL); > BSA (219 ± 3 U/mL); > haemoglobin (216 ± 9 U/mL) > egg albumin (210 ± 7 U/mL) > gelatin (0.0 U/mL). The gelatin was very slowly degraded by this enzyme even after 10 h incubation it showed poor proteolysis (16.6 U/mL). The results were in consistent with recent study about *B. subtilis* which reported the protease which unable to digest gelatine (Si et al., 2018).

The capability of this protease to utilize wide range of proteinaceous food substances like casein, egg albumin, haemoglobin, and soybean flour emphasizes its utility in enzymatic protein lysate preparation from milk, egg, meat, soybean flour. Although, the enzyme showed poor activity against gelatin, it could be a positive gain for specific proteolysis without impairing gelatin present in food as gelling agent. The gelatin used in wide range of food products as gelling agent due to its elasticity and clarity (Saha and Bhattacharya, 2010). The protease of *B. subtilis* AU-2 hence used for precise proteolysis application without upsetting the consistency of gelatin-stabilised food preparation.

4. Conclusion

The *Bacillus subtilis* AU-2 strain investigated in this study is an insect commensal and could be suitably used in food sector for food grade protease production like other *Bacillus subtilis* strains reported earlier. The improved production of protease by *B. subtilis* AU-2 in medium containing soybean meal (nitrogen source) revealed that the strain has been well adapted to utilise the soy bean seed proteins. The protease of *B. subtilis* AU-2 was found active in ambient physical parameters (pH, temperature) emphasises its utility in food applications. The metal ion reliant feature of metallo-protease from *B. subtilis* AU-2 and its capability to remain active in presence of various inorganic salts also provides assurance of activity and/or stability of this biocatalyst in the presence of various salts usually present in food.

The most significant findings includes the capability to catalyse various proteinaceous substrates like casein, egg albumin, haemoglobin, and soy bean flour, this accentuates its utility in enzymatic protein lysate preparation from milk, egg, meat, and soybean flour. The poor enzymatic activity of this protease against gelatin could be precisely utilize for achieving organized protein digestion without impairing the gelatin content which further assists to maintain consistency of gelatin-stabilised food preparation. Also, the capability to withstand H_2O_2 by protease of *B. subtilis* AU-2 advises its utility in improving/digesting the peptide contents of H_2O_2-preserved proteinaceous food. For food applications, the safety of the production strain is the most crucial factor; hence the protease of newly isolated insect commensal *Bacillus subtilis* AU-2 could be effectively used in food sector. The noteworthy outcomes of food related applications were also investigated which will be documented in subsequent study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101122.

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