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Elucidation of Structure of MALDI-TOF Peptide Fingerprint of Purified Microbial Protease and its use in Bioactive Dipeptide Precursor Synthesis

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ABSTRACT: Bioinformatic analysis of MALDI-ToF results of a purified protease of mutant of *Bacillus* sp. served as an important tool for providing detailed idea of structural and functional aspects of enzyme and making this specific for its applicatiom. Sequence homology using BLAST search showed the relatedness of protease of mutant *Bacillus* strain with protease of *Metarhizium robertsii* ARSEF 23 and its wild strain with zinc metalloprotease of *Kosmotoga*. Secondary structure prediction was done using Psipred software. Further this purified enzyme was utilized for synthesis of precursor of bioactive peptide which have positive impact on body functioning. Varieddipeptide precurosrs of bioactive peptides viz. dynorphin, aspartame and endomorphin were synthesized using purified mutant protease enzyme with 40mM of carboxyl and 60mM of amino component of different amino acids used. This enzymatic method resulted in 99 % conversion of reactant to dipeptide precursor of dynorphin with a yield of 104.35 mg. Dipeptide precursor of endomorphin showed 69 % conversion of reactant to product with a yield of 73.49 mg and 83 % conversion to dipeptide precursor for aspartame with 68.99 mg of yield respectively.

Keywords: Purified protease, Mutant and wild *Bacillus* sp., MALDI-ToF fingerprint, Bioinformatic analysis, Bioactive peptide synthesis, Dynorphin, Endomorphin

Abbreviations: MALDI-ToF, Matrix-Assisted Laser Desorption/Ionization-Time of Flight; BLAST, Basic Local Alignment Search Tool; BOC-, tert-butoxycarbonyl protecting group; HPLC, High performance Liquid chromatography; RT, retention time.

I. INTRODUCTION

Bioinformatics analysis serve as a major computational tool for functional and structural study of protein in detail using multiple sequence alignments, prediction of secondary and tertiary structures, catalytic sites and also protein fold recognition [1]. Various bioinformatics tools are used for analysis of these properties of protein making them specific for use in various industries for the synthesis of specific products. Microbial proteases are used in vast number of industrial applications and synthesis of precursor of bioactive peptide by purified protease enzyme is one of the major applications of enzymes which are highly specific [2-4]. Bioactive peptides are referred as specific protein fragments in which carboxyl group of one amino acid residue is coupled by peptide bond to the amino group of another amino acid residue [5-8]. These bioactive peptides possess positive impact on body functions thus, influencing human health and are considered as new generation of biologically active compounds showing drug and hormone like activities, used equally in the treatment of various medical conditions and consequently increasing the quality of life [9-10]. At present the functional foods and nutraceuticals are gaining high attentions in prevention of certain diseases thus promoting health [11]. Various technologies have been reported for the production of peptides, involving extraction from natural sources [12], through recombinant DNA technology [13], production from transgenic animals [14], production in cell free expression system [15] by chemical synthesis and through enzymatic synthesis using protease enzyme in synthesis of amide bonds [16]. A lot of peptides like lysine sweet peptide, kyotorphin, angiotensin, aspartame, enkephalin and dynorphinhave been synthesized successfully by using enzymatic approach [17-20] and small peptides can even be synthesized at industrial scale by using efficient enzyme reactors [20-23].

The aim of present study was to use bioinformatics tools with MALDI-ToF results of purified protease of mutant *Bacillus* sp. [21-22] for detailed structural and functional analysis and further use this purified protease for the synthesis of dipeptide precursor of selected bioactive peptides.

II. MATERIALS AND METHODS

A. Bioinformatics study of purified protease of wild and mutant of Bacillus sp.

In this study sequence of purified protease of mutant and wild *Bacillus* sp. obtained after MALDI-ToF analysis [24] was used for further analysis using bioinformatics tools. Protein sequence homology BLAST search was

performed with peptide fragments of mutant and wild strain against NCBI (http://ncbi.nlm.nih.gov/BLAST/) and then multiple sequence alignment was done using MEGA 6.0 software.

Secondary structure of purified protease peptide finger prints of wild and mutant of *Bacillus* sp. obtained after MALDI-ToF was predicted using Psipred software and tertiary structure was predicted by submitting sequences of purified proteins to SWISS-MODEL using (https://swissmodel.expasy.org/) server.

B. Bioactive peptide precursor synthesis using purified protease of mutant of Bacillus sp.

Different protected amino acids (BOC-L-Aspartic acid, L-Phenylamineesterhydrochloride, BOC-L-Proline, L-Tryptophanmethylester, BOC-L-Leucine, Glycineethylesterhydrochloride, BOC-D-Phenylamine and L-Leucinamidehydrochloride) with one protecting group (HiMedia) were used in the present study.

In this study purified protease of mutant of *Bacillus* sp. was used for the dipeptide precursor synthesis of bioactive peptides viz. dynorphin, aspartame and endomorphin. For the enzymatic reaction 40mM carboxyl component and 60mM amino component were dissolved in 2ml acetonitrile (containing 50 mm TAPS/NaOH buffer, pH 7.0) and then 10µl purified protease of mutant strain were added to each test. This mixture was placed in temperature controlled water bath at 50°C. Peptide synthesis was observed by noting the retention time of product formed against control by HPLC analysis using Reverse phase column (DEAE-Sepharose column 22×1.5 cm with 6cm matrix load). For analysis the reaction samples were dissolved in acetonitrile and a 10 µl of it was injected into HPLC. The solvent system/mobile phase comprised of acetonitrile: water in the ratio of 70:30 at a flow rate of 1 ml/min for 10 min. The synthesis of dipeptide was determined at 210 nm using UV detector in HPLC. The yields were calculated from the peak areas by the external standard method.

III. RESULTS AND DISCUSSION

A. Sequence homology of purified protease peptide fragments obtained after MALDI-ToF

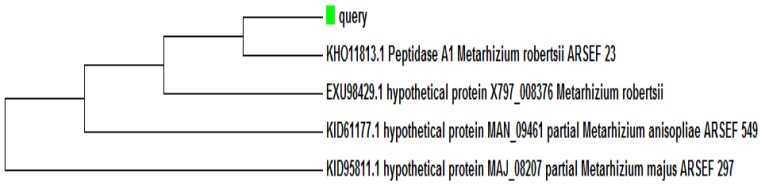
The protease of mutant and wild *Bacillus* sp. was purified and processed for MALDI-ToF analysis [24]. The sequences of purified peptide fragments obtained after MALDI-ToF were used for further bioinformatics analysis. To study the protein sequence homology BLAST search was performed with peptide fragments of mutant and wild strain obtained with MALDI-ToF against NCBI (http://ncbi.nlm.nih.gov/BLAST/) and then multiple sequence alignment was done using MEGA 6.0 software for analysing the relatedness of query sequence with known sequences. Fig. 1 shows the relatedness of protease of mutant strain with peptidase of *Metarhizium robertsii* ARSEF 23 and of wild strain with zinc metalloprotease of *Kosmotoga*.

B. Structure prediction of mutant and wild Bacillus sp. peptide fingerprints

Secondary structure prediction of purified protease peptide finger prints obtained after MALDI-ToF study was done in both wild and mutant strains using Psipred software. Secondary structure of a protein gives detailed information about its alpha helix, beta strands, and loops in the structure. This analysis showed that mutation has not distorted the protein structure in mutant strain and its functional units i.e. alpha-helix, beta-strand and loop were equally intact even after mutation in the mutant. Fig. 2 shows the intact secondary structure of purified protease of both mutant and wild strain.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Peptidase A1 [Metarhizium robertsii ARSEF 23]	151	151	100%	3e-46	100%	XP_011410777.1
hypothetical protein X797_008376 [Metarhizium robertsii]	151	151	100%	8e-46	99%	EXU98429.1
hypothetical protein MANI_114238 [Metarhizium anisopliae]	81.6	81.6	100%	2e-18	88%	KFG86087.1
hypothetical protein MAJ_08207 [Metarhizium majus ARSEF 297]	72.4	72.4	37%	1e-13	97%	KID95811.1

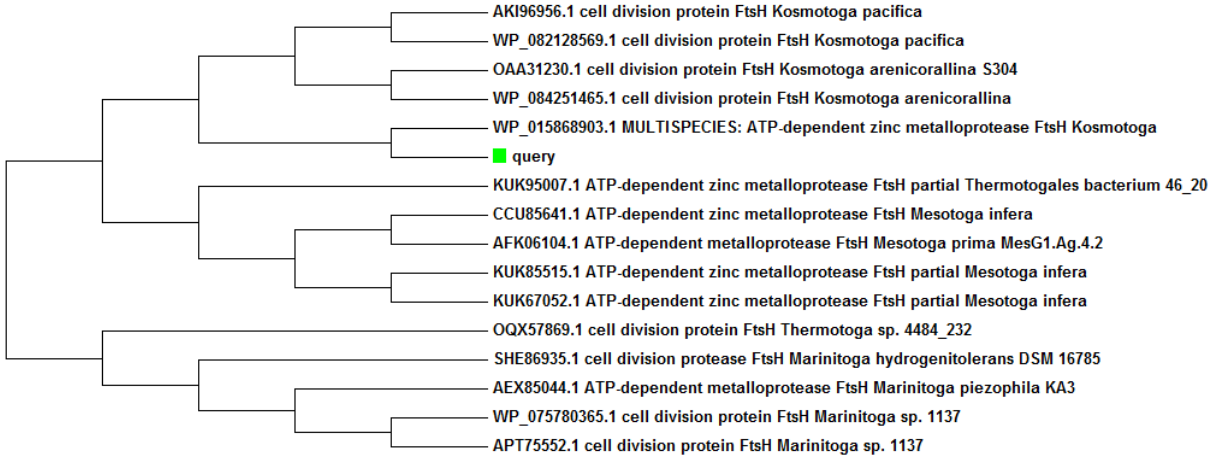
A



B

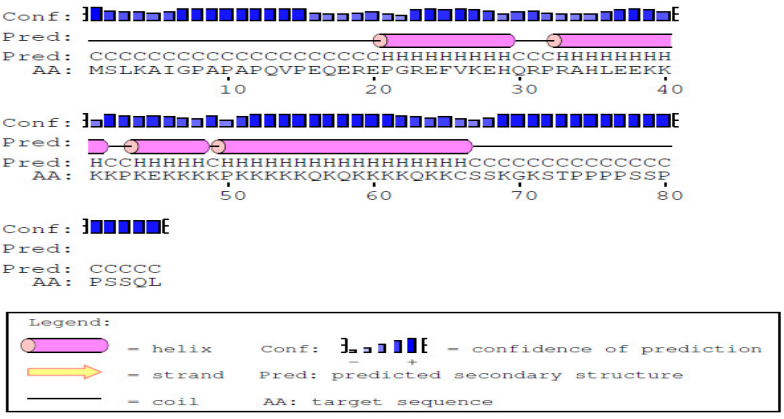
Description	Max score	Total score	Query cover	E value	Ident	Accession
MULTISPECIES: ATP-dependent zinc metalloprotease FtsH [Kosmotoga]	1311	1311	100%	0.0	100%	WP_015868903.1
cell division protein FtsH [Kosmotoga pacifica]	1056	1056	98%	0.0	80%	AKI96956.1
ATP-dependent metallopeptidase FtsH/Yme1/Tma family protein [Kosmotoga pacifica]	1043	1043	97%	0.0	80%	WP_082128569.1
cell division protein FtsH [Kosmotoga arenicorallina S304]	1021	1021	95%	0.0	80%	OAA31230.1
ATP-dependent metallopeptidase FtsH/Yme1/Tma family protein [Kosmotoga arenicorallina]	1007	1007	94%	0.0	80%	WP_084251465.1
ATP-dependent metallopeptidase FtsH/Yme1/Tma family protein [Mesotoga sp. B105.6.4]	1006	1006	95%	0.0	77%	WP_103133927.1
ATP-dependent metallopeptidase FtsH/Yme1/Tma family protein [Mesotoga sp. Brook.08.105.5.1]	1006	1006	95%	0.0	77%	WP_099830666.1
ATP-dependent zinc metalloprotease FtsH [Mesotoga infera]	1003	1003	95%	0.0	77%	CCU85641.1
ATP-dependent metallopeptidase FtsH/Yme1/Tma family protein [Mesotoga prima]	1003	1003	95%	0.0	77%	WP_014730230.1
ATP-dependent metallopeptidase FtsH/Yme1/Tma family protein [Mesotoga sp. H07.pep.5.3]	997	997	95%	0.0	76%	WP_099771863.1
ATP-dependent zinc metalloprotease FtsH [Thermotogales bacterium 46_20]	967	967	99%	0.0	73%	KUK95007.1
ATP-dependent zinc metalloprotease FtsH, partial [Mesotoga infera]	904	904	87%	0.0	77%	KUK85515.1
ATP-dependent zinc metalloprotease FtsH, partial [Mesotoga infera]	902	902	87%	0.0	77%	KUK67052.1
cell division protein FtsH [Thermotoga sp. 4484_232]	781	781	92%	0.0	63%	OQX57869.1

C

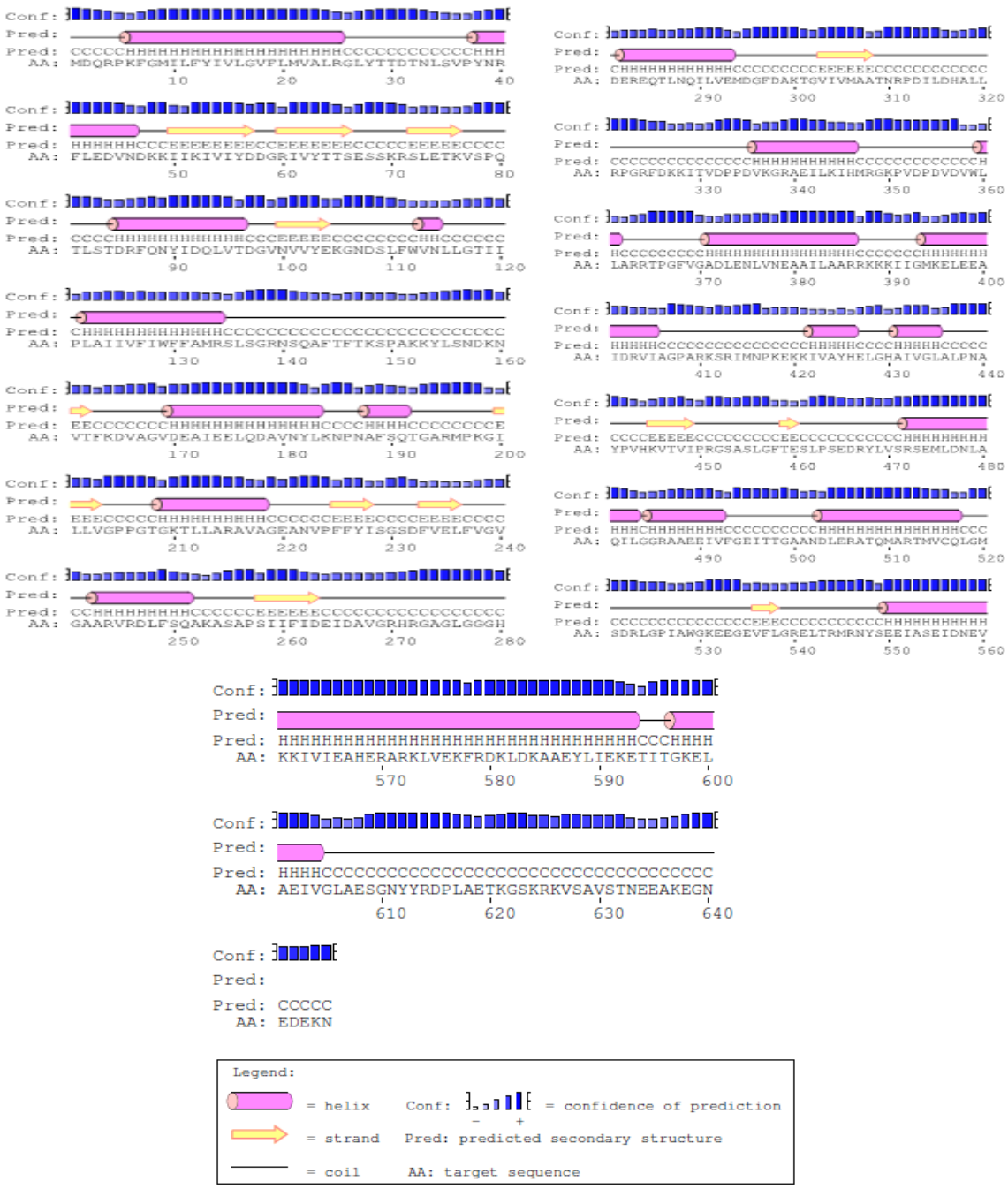


D

Fig. 1. BLAST of protease of mutant *Bacillus* sp. with related protein in Protein Data Bank. A) showing highest similarity and query coverage with structure of *Metarhizium robertsii* ARSEF 23. B) Cladogram of peptide fragments showing similarity of mutant *Bacillus* sp. with related protein. C) BLAST of protease of wild *Bacillus* sp. with related protein in Protein Data Bank showing highest similarity and query coverage with zinc metalloprotease of *Kosmotoga*. D) Cladogram of peptide fragments of wild strain showing similarity with zinc metalloprotease of *Kosmotoga*.



A



B

Fig. 2. Prediction of the secondary structure of purified protease using Psipred software. A) mutant and B) wild *Bacillus* strain.

Prediction of tertiary structure of purified protease of mutant strain was done by submitting sequences of purified proteins to SWISS-MODEL. This model predicted the active site of catalytic triad (Ser, Asp and His) in both wild protease and peptidase from mutant strain of *Bacillus* sp. As the peptide fingerprint sequence of mutant strain obtained after MALDI-TOF analysis is small and shows only ser in active side in peptidase. These observations indicated that even after mutation some variation occurred in the amino acid sequence but still mutant strain retained its active site as it was present in the wild strain (Fig. 3).

C. Biologically active dipeptide precursor synthesis using purified proteases

The use of purified protease of wild strain of *Bacillus* sp. is already reported [25].

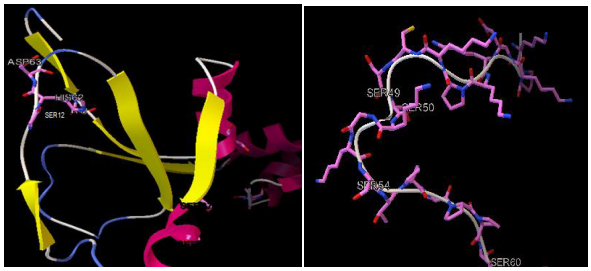
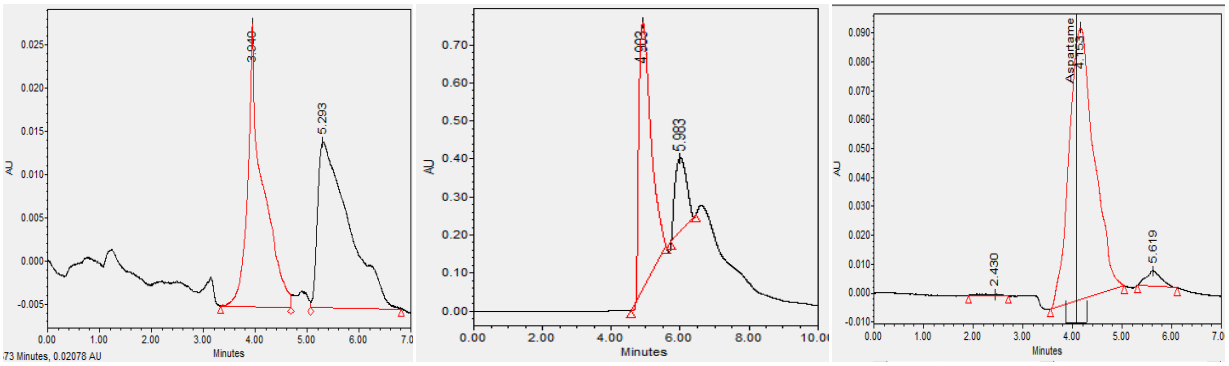


Fig. 3. 3-D protein structure model of wild and mutant strains of *Bacillus* sp. showing active site of catalytic triad (Ser, Asp and His). A) wild protease and B) mutant peptidase of *Bacillus* sp. in SWISS-MODEL using https://swissmodel.expasy.org/ server.

In this study only the results of purified protease of mutant of *Bacillus* sp. for the synthesis of dipeptide precursors of bioactive peptides using different amino acids with one end protective are discussed. In this assay reaction carboxyl component of one amino acid reacted with amino terminal of another amino acid and the retention time for synthesized dipeptide precursors viz., endomorphin, dynorphin and aspartame were found to be 3.940, 4.903 and 4.153 respectively as shown in Table 1 and Fig. 4. The results were compared with the standards of each bioactive peptides reported in literature for its confirmation. The synthesized dipeptide product peaks showed similarity with reported range [25-26]. The present study resulted in 99 % conversion of reactant to dipeptide precursor of dynorphin product in reaction mixture with a yield of 104.35 mg, and 69 % conversion of dipeptide precursor of endomorphin reactant to product with a yield of 73.49 mg. In third case the precursor of aspartame formed showed 83 % conversion of reactant into product with 68.99 mg of yield.

Table 1: Synthesis of different bioactive peptides dipeptide precursors using purified protease of mutant of *Bacillus* sp.

Carboxyl terminal group used	RT (min)	Amino terminal group used	RT (min)	Dipeptide product synthesized	RT (min)	Conversion (%)	Amount synthesized (mg)
BOC-L-Proline	4.284	L-Tryptophane ME	5.109	Endomorphins	3.940	69	73.49
BOC-D-Phenylanine	4.748	L-LeucinamideHCl	4.966	Dynorphins	4.903	99	104.35
BOC-L-Asp. acid	2.723	L-PhenylesterHCL	4.635	Aspartame	4.153	83	68.986



A			B			C		
RT (min)	Area (μV^2Sec)	Height (μV)	RT (min)	Area (μV^2Sec)	Height (μV)	RT (min)	Area (μV^2Sec)	Height (μV)
3.940	647261	32735	4.903	17203527	699425	4.153	3285154	93980

Fig. 4. HPLC detection of dipeptide precursors of bioactive peptides viz. A) endomorphin.B) dynorphin and C) aspartame synthesized with purified protease of *Bacillus* sp. using varying combination of carboxyl component and amino component under standard assay conditions.

In literature a large number of reports on the homology of proteases are available and in computational molecular biology these protein sequence homology are important for a variety of purposes. Using a nucleotide and amino acid sequences of enzymes, structural-functional relationship of enzymes can be analyzed [28-29]. A lot of peptides have been synthesized successfully by using enzymatic methods as reported earlier [17-20, 30]. Some small peptides even can be synthesized at the commercial scale by using efficient enzyme reactors [29]. Microbial proteases are advantageous over the use of chemical means of

peptide synthesis [31]. For the synthesis of peptide bond, proteases are selected on the basis of specificity against each amino acid residue in the reaction [32]. Therefore, search for use of new microbial proteases could help in synthesis of new and novel combination of bioactive peptides.

IV. CONCLUSION

Bioinformatics analysis of protease of mutant of *Bacillus* sp. resulted in detailed structural information of this enzyme. This study revealed that mutation did not show any negative effect on the structure and functionality of this protease on comparing it with the wild strain. These finding showed that mutant protease might also find its use in specific applications for dipeptide precursor synthesis of bioactive peptides. Protease enzyme of mutant strain was able to synthesize precursors of three bioactive peptides viz., dynorphin, endomorphin and aspartame. Aspartame is utilized as the artificial sweetner agent and dynorphin and endomorphin are used as pain relieving agents.

V. FUTURE SCOPE

This purified protease of mutant of *Bacillus* sp. can also be used for the synthesis of bioactive peptide precursor of other biologically active peptides viz., cerulin, sweet lysine peptide, somatostatin, vessopressin, cholecystokinin and skin growth promoter stimulating hormone etc. [31-32].

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Conflict of Interest: The authors declare that they have no conflict of interest

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