www.ijpbs.net

INTERNATIONAL JOURNAL OF PHARMA AND BIO SCIENCES

Internationally indexed journal

Indexed in Chemical Abstract Services (USA), Index coppernicus, Ulrichs Directory of Periodicals, Google scholar, CABI ,DOAJ , PSOAR, EBSCO , Open J gate , Proquest , SCOPUS , EMBASE ,etc.

Rapid and Easy Publishing

The "International Journal of Pharma and Bio Sciences" (IJPBS) is an international journal in English published quarterly. The aim of IJPBS is to publish peer reviewed research and review articles rapidly without delay in the developing field of pharmaceutical and biological sciences

Pharmaceutical Sciences

- Pharmaceutics
- Novel drug delivery system
- Nanotechnology
- Pharmacology
- Pharmacognosy
- Analytical chemistry
- Pharmacy practice
- Pharmacogenomics
- Polymer sciences
- Biomaterial sciences
- Medicinal chemistry
- Natural chemistry
- Biotechnology
- Pharmacoinformatics
- Biopharmaceutics

Biological Sciences

- Biochemistry
- Biotechnology
- Bioinformatics
- Cell biology
- Microbiology
- Molecular biology
- Neurobiology
- Cytology
- Pathology
- Immunobiology

ELSEVIER

***Indexed in Elsevier Bibliographic Database**

For "Instruction to Authors" visit **www.ijpbs.net**

For any Queries, email to **"editorijpbs@yahoo.in"**

ISSN 0975-6299 Vol 3/Issue 1/Jan – Mar 2012

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE

MICROBIOLOGY

DIFFERENT ASPECTS OF PRODUCTION OF THERMOLYSIN-LIKE PROTEASE FROM *THERMOACTINOMYCES THALPOPHILUS*

D.R MAJUMDER AND P.P KANEKAR *

Microbial Sciences Division, MACS' Agharkar Research Institute, G.G. Agarkar Road, Pune-411004, India

P.P KANEKAR
Microbial Sciences Division, MACS'Agharkar Research Institute, G.G. Agarkar Road, Pune-411004, India

***Corresponding author**

ABSTRACT

Thermoactinomyces thalpophilus (Gene Bank accession number EF 397000) isolated from the waters of a thermal spring situated in Western Ghats, Maharashtra, India was found to be a potent producer of thermolysin-like protease (TLP). TLP was capable of bringing about condensation/ biotransformation of two amino acids, viz. Z-L-aspartic acid and L-phenylalanine methyl ester into aspartame, which is an artificial, non-saccharide sweetener used as a sugar substitute in some foods and beverages. The maximum yield 181 U/ml/min was obtained at 55^{o}C in glucose yeast extract medium(pH 7.2) containing $CaCl_2$ (0.2 % w/v) with an inoculum size of 0.5% v/v after 18 hours incubation under shake-culture conditions (130 rpm)

KEY WORDS

TLP, biotransformation, aspartame, *Thermoactinomyces thalpophilus*., Thermolysin

INTRODUCTION

Thermophiles are the most primitive organisms which are important biotechnologically for thermostability, less incubation time, early sporulation and immense industrial feasibility. Habitats of thermophiles are mostly pristine-like thermal springs and meteorite craters[1-5].

Thermophilic bacteria are known to produce novel bioactive molecules like Taq polymerase[6], thermolysin[7] and biolysin[8-9] wherein the latter two are thermostable proteases.

Thermoactinomycetes constitute an extensive and diverse group of Gram-positive, aerobic, largely mycelial bacteria, many of which are commercially important in the production of bioactive molecules specially protease enzymes[5, 10-14]. Among these, Thermolysin[7] is a neutral zinc-metalloendopeptidase which has immense application in protein sequencing, peptide mapping and protein structural analysis[15-16]. The enzyme brought about a revolution in industrial enzymology since 1979 when Isowa *et al* showed the enzymatic peptide synthesis of the artificial sweetener, viz. aspartame, through biotransformation/ condensation reaction of two amino acids involving Z-L-Aspartic acid and L-Phenyalanine methyl ester by thermolysin. Since then several thermolysin-like protease (TLP) enzymes have been isolated from various sources[17].

The present study was undertaken to explore new sources for TLP since there have been no reports of TLP producing 'aspartame' from *Thermoactinomyces thalpophilus* as far as we are aware. This paper deals with identification of the isolate producing TLP and confirmation of the production of aspartame as well as optimization of the environmental parameters for maximum production of the protease.

MATERIALS AND METHODS

Isolation of the bacterial cultures:

Thirty-four isolates from three different ecosystems (Hotspring from Western Maharashtra, Lonar lake and Pashan lake) deposited in MCM (WDCM code 561) culture collection were screened for protease production on CGYP agar (Casein 1%; glucose 1%; yeast extract 0.5%; peptone 0.5%; NaCl 0.5%; $CaCl_2$ 0.2%; pH 7.4; agar 2%) according to the modified Cowan's method (Cowan *et al.* 1982). All isolates were also grown in GYP broth 50 ml in 250 ml flask (130 rpm) at 55^{o}C for 24 hours to estimate the enzyme activity (U/ml/min).

The zone of clearance around the colonies of the isolates due to casein hydrolysis on CGYP agar plates was measured as a ratio of diameter of the clear zone and that of the colony on CGYP. The protease activity was measured according to modified Anson's method (1938) as stated[18]. The protease activity in the liquid medium was compared with that in the solid medium. The strain showing maximum activity in both the media was selected for further studies.

Characterization and Identification of isolates:

One isolate PD1 was selected for further studies based on its protease production ability. Microbiological properties were investigated according to the morphological and biochemical tests described[14, 19]. Genus and species confirmation was carried out by 16Sr RNA sequence analysis[20].

Enzyme Assay

Enzyme Assay: [18, 21]

1 ml of Hammersten Casein (0.625% stock)

Prepared in 50mM Tris HCl buffer (pH 7.5) containing 5mM $CaCl_2$ was used. This was incubated at 55°C for 10 minutes in water bath. A 100µl cell free broth (Enzyme) suitably diluted with 150 µl of buffer solution 50mM Tris HCl (pH 7.5) containing 5mM $CaCl_2$ buffer was added to the substrate solution and incubated at 55°C for 30 minutes in water bath. The reagent blank was prepared by replacing the enzyme solution with the buffer in above protocol. The reaction was stopped with 1.25 ml of 5% Trichloroacetic acid and the mixture was incubated at 55°C for 15 minutes in water bath. An enzyme blank was prepared by mixing buffer, enzyme, TCA and substrate in the identical order. The white precipitate formed was removed by centrifugation at 8,000 rpm for 10 minutes at 6°C. The supernatant was subjected to further estimation. 0.1 ml of supernatant was added to 0.9 ml of distilled water mixed with 0.5 ml of alkaline copper tartarate solution and the mixture was incubated for 10 minutes at room temperature. A 2 ml of Folin Ciocalteau Reagent (1.15 dilution) was added and incubated for 10 minutes at room temperature[22]. The absorbance was measured at 660nm. One unit of protein activity was defined as the amount of enzyme, which liberated 1µg of tyrosine in one minute at 55°C.

Protein content: Protein content was measured with BSA as standard protein by Lowry's method[22]. 1ml of fermentation broth (enzyme) was mixed with 1ml of 5% TCA and was incubated for 20 minutes at room temperature. Precipitate formed was removed by centrifugation at 8,000 rpm for 10 minutes at 6°C. The supernatant was discarded. The precipitate was taken and dissolved in 1 ml of 0.1N NaOH solution. 0.1 ml of this solution was added to 0.9 ml of distilled water, to which 0.5 ml of alkaline copper tartarate solution was added and the mixture was incubated for 10 minutes at room temperature.
2 ml of Folin Ciocalteau reagent (1.15 dilution) was added and incubated for 20 minutes at room temperature. The absorbance was measured at 660nm. Protein content was expressed as milligram of protein per milliliter of fermentation broth or cell free extract (enzyme)

$$\text{Specific activity was calculated as} = \frac{\text{Units of Enzyme/ml/min}}{\text{mg of protein/ml}}$$

Biotransformation/ Condensation Reaction
Aspartame production was measured according to method of Isowa[23]. The method has three steps:

I. Production of Aspartame precursor enzymatically[23]:
Z-L-Aspartic acid (533 mg) along with L-Phenyalanine methyl ester (861 mg) was mixed with 10ml of Milli Q water (pH 6-8). A 1 ml of crude enzyme was added to above mixture and the solution was incubated at 55°C for 1 hour.

II. Deprotection of aspartame precursor[24]:
The white precipitate (i.e. aspartame precursor) was taken in a round bottom glass bottle to which were added 90% acetic acid (CH_3COOH) and 2-3mg of Pd catalyst. The bottle was kept on a PARR shaker at room temperature and 50-psi pressure was applied (H_2 gas) for 4h.

III. Detection of aspartame by HPLC (NIOSH, 1994):
C- 18 column, Detection – UV, 210nm, Mobile phase-85:15 of 0.1% TFA: Acetonitrite (isocitrate) Flow rate- 0.8ml/min.

Relation of Protease production with growth curve
In order to determine relation between growth of the organism and the protease production a sample of GYE medium (200 ml) containing (%w/v) glucose (3), yeast extract (1), NaCl (0.5) and $CaCl_2$ (0.2) taken in 1000ml flask was inoculated with 0.5% inoculum of spore suspension of the strain PD1 and incubated at 55°C, on a orbital shaker incubator (130 rpm) for 24 h. Aliquots (1 ml each) were taken at regular intervals of 2 h , and centrifuged at 10,000g for

10 minutes. The total cell protein was estimated by Lowry's method after lysing the cells in pellet with 10% KOH at 70°C. The extracellular protease activity of the cell free broth was estimated.

Effect of medium composition on Protease production

To select a basal medium for optimum protease production, eleven different media were inoculated at 55°C under shaker culture condition for 24 hours and the protease activity of the CFB was estimated. The medium yielding maximum protease activity was selected for further modifications.

The composition of 11 different media (composition in %w/v)

1. MCY (maltose Casamino acid yeast extract medium) Maltose (1), Casamino acid (0.5), Yeast extract (0.04), K_2HPO_4 (0.5), NaCl (0.2), pH 7.2, $CaCO_3$ (1.5)
2. CYC (Czapek-Dox yeast extract Casamino acid medium) Sucrose (3), $NaNO_3$ (0.2), K_2HPO_4 (0.1), $MgSO_4$ (0.05), KCl (0.05), $FeSO_4$ (0.01), Yeast extract (0.2), Casamino acid (0.6) pH 7.2
3. CD (Sucrose Casamino acid Casein peptone medium) Sucrose (1), Casamino acid (0.15), Casein (0.15), NaCl (0.1), K_2HPO_4 (0.12), $Na_2HPO_4.2\ H_2O$ (0.28), $FeSO_4.\ 7H_2O$(0.001), pH 7.2, $CaCO_3$ (0.0005),
4. CSL (Corn steep liquor) CSL (0.5), Sucrose, Casamino acid (0.3), Corn Starch (0.5), $NaNO_3$ (0.1), NaCl (0.25), KCl (0.0025), FeSO4. $7H_2O$(0.0005), Magnesium glycerol phosphate (0.25), pH 7.2, $CaCl_2.2H_2O$ (0.025),
5. CBS (Sucrose ammonium sulphate yeast extract) Sucrose (5), $(NH_4)_2SO_4$ (0.4), yeast extract (0.3), K_2HPO_4 (0.13), $MgSO_4.7H_2O$ (0.002), $ZnSO_4.\ 7H_2O$(0.001), $FeSO_4.\ 7H_2O$(0.001), $MnSO_4.\ 7H_2O$(0.001) , $CuSO_4.\ 7H_2O$(0.006), pH 7.2, $CaCO_3$ (0.3)
6. MM (Minimal medium) L-aspargine (0.05), K_2HPO_4 (0.05), MgSO4.7H2O (0.02), $FeSO_4.\ 7H_2O$ (0.001), glucose (1), pH 7.2
7. HMM (glycerol arginine) Glycerol (0.2), L-arginine (0.25), NaCl (0.1), $MgSO_4.7H_2O$ (0.01), $FeSO_4.7H_2O$(0.01), pH 7.2, $CaCO_3$ (0.01)
8. SC (Starch Casein medium) Starch (1), Casein (0.03), KNO_3 (0.2), NaCl (0.2),K_2HPO_4 (0.2), $MgSO_4$ (0.005), $FeSO_4$(0.002), pH 7.2
9. PY (Peptone yeast extract medium) Peptone (2), yeast extract (2), glycerol (0.2), $MgSO_4.7H_2O$ (0.03), pH 7.2
10. NCCP/GYP-mentioned earlier in the paper
11. GYE-mentioned earlier in the paper

Different concentrations of carbohydrate and nitrogen source of the chosen media GYE were taken to determine the ideal concentration ratio for maximum protease activity. Original GYE had 3% glucose and 1% yeast extract. At first the yeast extract concentration was kept constant while the concentration of glucose was changed- Glucose: YE, 1:2, 2:1, 3:1, 4:1, 5:1. Then, concentration of glucose was kept constant while yeast extract concentration was altered- Glucose: YE, 3:0.5, 3:1, 3:2, 3:3, 3:4. (In all cases, 50ml of medium in 250ml flasks were inoculated with 0.5% and the flasks were incubated at 55°C, on orbital shaker incubator (130 rpm) for 24 hours).

Different concentrations of carbohydrate and nitrogen sources were used to determine their effect on protease production. PD1 was grown in GYE medium containing carbon sources (1% and 3% w/v) e.g. Sucrose, fructose, maltose, soluble starch and glycerol. Similarly yeast extract was substituted by other nitrogen sources (1% w/v) e.g. Peptone, Beef extract, Casein, Soybean meal, Tryptone, Soya flour, Wheat bran, Ragi, Bengal gram flour, $NaNO_3$, NH_4Cl and $(NH_4)_2SO_4$.

The concentrations of NaCl in GYE were also altered to determine the ideal concentration for production of protease. 0.5, 1 and 5% (w/v). Even the type of calcium salt in GYE medium i.e. $CaCO_3$ and $CaCl_2$, in their different concentration was examined to determine the ideal salt with its

concentration that produced maximum protease. The range of concentration for $CaCO_3$ were 0.1, 0.3, 0.6, 1 and 1.5 (%w/v) and for $CaCl_2$ were 0.02, 0.05, 0.1, 0.15 and 0.2 (%w/v). Other trace salts were checked for their effect on the protease production. GYE medium was fortified with trace salt solution (0.1 %w/v), K_2HPO_4, $FeSO_4$. $7H_2O$, $MnCl_2$ and $ZnSO_4$. Effect of detergents on protease production was observed by adding some surface active agents like sodium perborate, Tween 80, SDS, sodium tripolyphosphate (0.01 %w/v) to GYE medium. Lastly among media composition, free amino acids like casamino acid, arginine, aspargine, threonine and alanine (0.5 and 1 %w/v) were added to GYE to check their individual effect on protease production and the protease activity was assayed.

Effect of Biological parameters on protease production

To check the inoculum size on protease production firstly, spore stock (12.5 $x10^6$ spores/ml) of PD1 were used in different concentrations (0.5%,1%, 2%, 3%,4%, v/v) in GYE medium. Secondly, seed culture was prepared by inoculating 0.5% v/v of spore stock in 100ml of GYE medium in 500ml flask. After 10-12 hours, 100ml of fresh GYE medium was inoculated with different concentrations of seed culture ranging 1.5%-4.5% and the protease activity was determined after 24 hours. All the flasks were incubated at 55^{o}C, on a orbital shaker incubator (130 rpm)

Effect of Physical parameters on protease production

The effect of aeration and agitation on protease production was determined. Two 250ml flasks each having 50ml of GYE medium were inoculated (0.5% w/v) with PD1 spore stock, and kept under static condition at 55^{o}C and another at 130 rpm under shaking condition at 55^{o}C. Different volumes of GYE with 0.5% w/v inoculum like 50 ml, 100 ml, 150 ml and 200 ml were taken in 250 ml flask. The flasks were incubated at 55^{o}C, on a orbital shaker incubator (130 rpm). Lastly, different rpm like 100-150 rpm were set on the shaker at 55^{o}C for 50ml GYE (0.5% inoculum) in 250 ml flask. The protease activity was assayed after 24 hours.

To determine the optimum pH for the protease production GYE medium was prepared with different pH ranging from 6 to 9. PD1 was grown in each medium as mentioned above and the protease activity of the cell free broth was determined.

RESULT

The extracellular protease activity of thirty four isolates was determined qualitatively as the ratio of diameter of the clear zone and that of the colony on Casein glucose yeast extract peptone agar (CGYP) while quantitative estimation was carried out by determining the protease activity (U/ml/min) according to Ansons[18]. The total protein was estimated accordingly to Lowry's method. The activities exhibited by all thirty four isolates in liquid and solid media are compared in **Table 1** which showed that isolate PD1 was the highest producer of extracellular protease activity ($105Uml^{-1}$).

Table 1
Protease activity in GYP broth and on CGYP agar

Sr. No.	Isolate		Protease Activity A:B (Ratio of zone of clearance and colony)	Protease Activity (U / ml / min)	Total Protein (mg / ml)	Specific Activity
	Name	Source				
1	PD1	Hot Spring	3.4	105.55	3.29	32.08
2	PD2	Lonar lake	1.5	21.61	1.09	19.82
3	PD3	Lonar lake	0.7	10.31	0.52	19.82
4	PD4	Lonar lake	2.1	41.90	2.09	20.04
5	PD5	Pashan lake	1.8	30.98	3.2	9.68
6	PD6	Pashan lake	0.7	10.9	1.9	5.73
7	PD7	Pashan lake	1.8	32.67	6	5.44
8	PD8	Pashan lake	2	34.06	2.9	11.74
9	PD9	Pashan lake	1	12.09	1.85	6.2
10	PD10	Pashan lake	0.7	9.91	1.33	7.45
11	PD11	Pashan lake	0.8	11	1.4	7.85
12	PD12	Pashan lake	3	87.18	3.57	24.42
13	PD13	Pashan lake	2	37	5	7.4
14	PD14	Pashan lake	1.5	18	2.6	6.92
15	PD15	Pashan lake	2	37.06	4	9.26
16	PD16	Pashan lake	0.5	5.15	1.04	4.95
17	PD17	Pashan lake	0.4	4.02	1	4.02
18	PD18	Pashan lake	2.1	47.99	2.3	20.86
19	PD19	Pashan lake	1.7	27	1.8	15
20	PD20	Pashan lake	2	37.08	2.8	13.24
21	PD21	Pashan lake	3	88.85	3.7	24.01
22	PD22	Pashan lake	0.5	4.95	1	4.95
23	PD23	Pashan lake	2.3	49.98	1.28	39.04
24	PD24	Pashan lake	1.2	15.07	1.66	9.07
25	PD25	Pashan lake	1	11.7	0.76	15.39
26	PD26	Pashan lake	1.5	23	1.42	16.2
27	PD27	Pashan lake	1.8	31.7	3.7	8.56
28	PD28	Pashan lake	1.8	29	2.16	13.42
29	PD29	Pashan lake	1.8	29.3	2.5	11.72
30	PD30	Pashan lake	1.5	20	1.4	14.28
31	PD31	Pashan lake	3.1	97	4.9	19.97
32	PD32	Pashan lake	2.5	64	3.2	20
33	PD33	Pashan lake	3	87	4.8	18.12
34	PD34	Pashan lake	1.6	23.79	1.95	12.2

Extracellular protease activity from the isolate. PD1

The white precipitate formed from the crude concentrate was incubated with two amino acids, viz Z-L-Aspartic acid and L-Phenyalanine methyl ester respectively. The precipitate after de-protection[24] was identified as aspartame with the help of HPLC[25].

The comparison of retention time of the standard aspartame (Equal^{TM}) and the one produced by the protease enzyme from PD1 is included in **Table 2** and **Figures 1A and 1B** respectively.

Table 2
HPLC analysis of aspartame

Sample	Retention time
1.Product formed from the protease from PD1	9.90
2.Std. Equal(containing aspartame)	10.08

Figure. 1A
HPLC analysis of standard aspartame (Equal^{TM})

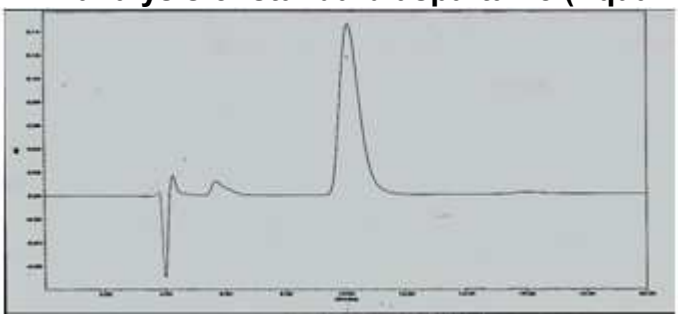
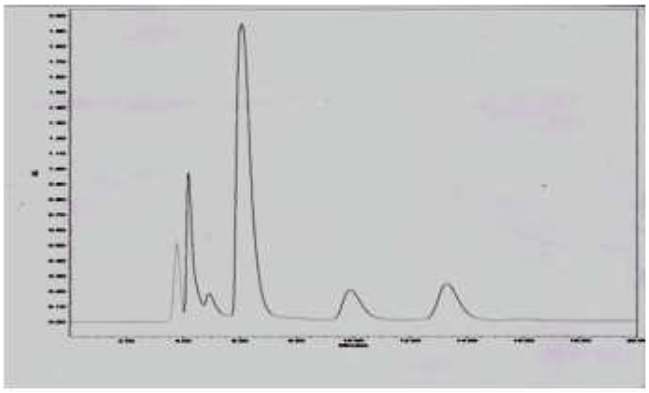
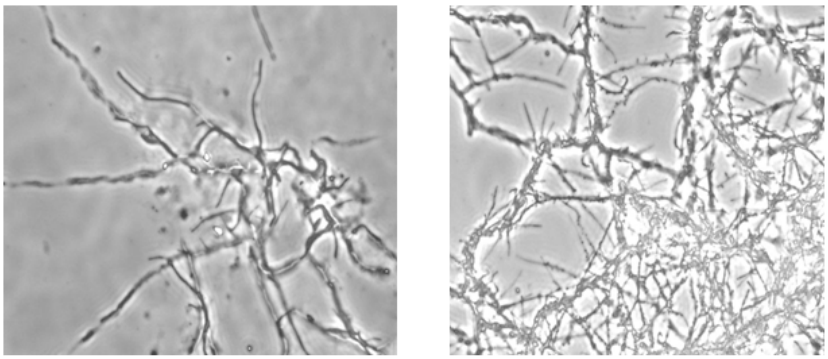


Figure. 1B:
HPLC analysis of aspartame from PD1 protease



The isolate was then identified with the help of various morphological, biochemical and molecular biology techniques. The microscopic observation of cover slip culture in **Fig 2A and 2B** shows the aerial mycelial spores of 16 hour old culture of PD1.



A B

Figure 2

A: Arial mycelium showing sessile spores and
B) Substrate and arial mycelia showing sessile spores. Cover slip culture preparation of PD1 observed under Phase Contrast Microscope, 400x.

Table 3 ***shows the morphological characterization of the PD1 colony.***

Table 3
Colony Characteristics of the isolates

Sr.No	Size mm	Shape	Margin	Elevation	Consistency	Opacity	Colour	Gram's Character
PD1	5	Round	uneven	flat	dry	opaque	white	+
PD12	7	Round	uneven	flat	dry	opaque	white	+
PD31	2	Round	uneven	raised	dry	opaque	white	+

This was followed by the biochemical characterization of the isolate of PD1 (**Table 4**) where the utilization of different nutrients and enzyme producing capacity was judged.

Table 4
Biochemical characteristics of the isolate

Biochemical tests	PD1	PD12	PD31
Growth at 55^0C			
(0.5 % NaCl)	+	+	+
(1% NaCl)	+	+	+
Growth at 30^0C			
30^oC (0.5% NaCl)	-	-	-
30^oC (1% NaCl)	-	-	-
Utilization of Carbohydrate			
Fructose	+	-	-
Mannitol	+	+	-
Sucrose	+ very less)	-	-
Trehalose	+	+	+
Hydrolysis of carbohydrates and proteins			
Starch	+	+	+
Casein	+	+	+
Gelatin	+	+	+

The morphological and biochemical tests were compared with the standard reference strain, which indicated that the culture PD1 resembled thermophilic actinomycetes and could belong to the genus *Thermoactinomyces.* To confirm the identity of the isolate, phylogenetic classification based on the analysis of nucleic acid sequence of 16sRNA was performed, which confirmed its similarity with Thermoactinomyces *thalpophilus.*

The production of protease with relation to growth of PD1, measured in terms of total protein content of biomass is explained in **Fig 3.**

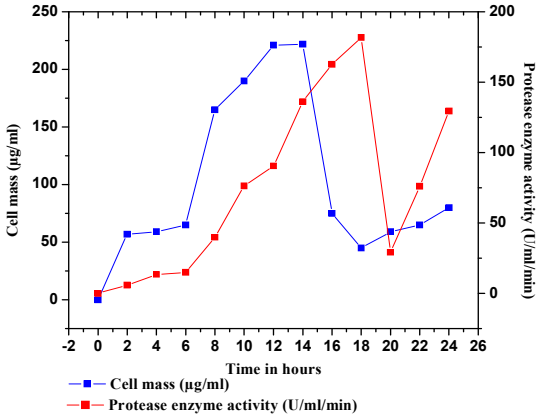


Figure 3
Growth curve showing the relation between growth of the organism and protease production.

The optimum protease activity was observed after 18 h incubation, when growth is minimum in terms of biomass. Maximum protease accumulation took place when the culture entered the stationary phase. Production of protease enzyme was determined in different media and yield of the enzyme in terms of activity is shown in **Fig 4.**

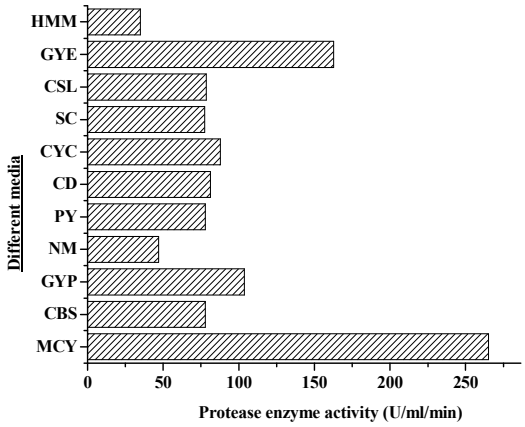


Figure 4
Protease enzyme activity in different media

The protease yield was maximum (265 U/ml/min) in MCY medium which had Casamino acid. Even the trace salt K_2HPO_4 at 0.1% concentration also influenced the production of the protease positively. However, GYE medium which gave 162 U/ml/min enzyme activity was

selected for further modification of the medium to enhance the yield due to cost considerations.

Effect of different carbon (at 3 % and 1 % concentration) and nitrogen sources on protease production are summarized in **Figures 5-7** respectively.

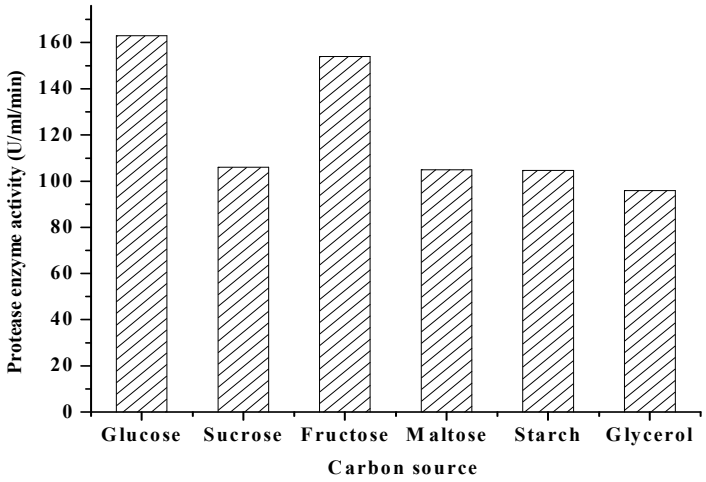


Figure 5
Protease enzyme activity with different carbon sources at 3% concentration

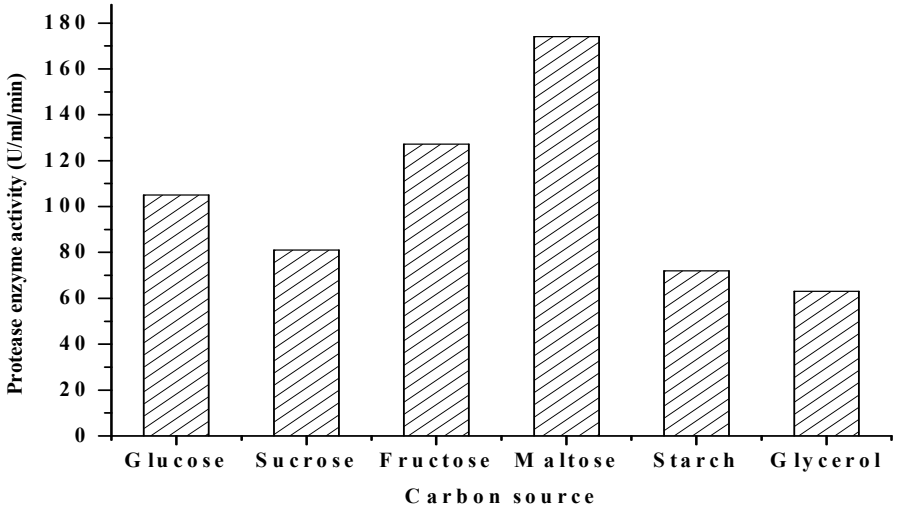


Figure 6
Protease enzyme activity with different carbon sources at 1% concentration

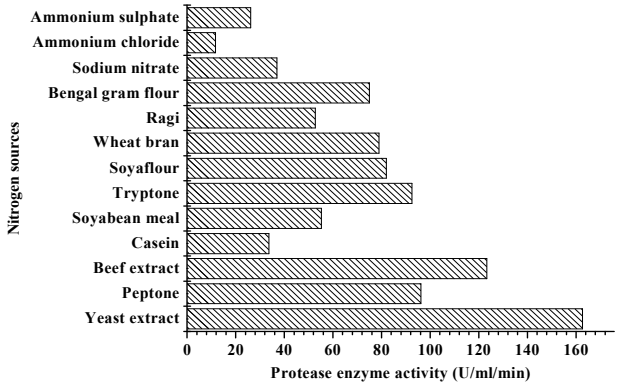


Figure 7
Protease enzyme activity with different nitrogen sources

Maltose and glucose seemed to be the logical choice for production of protease. Maltose at concentrations higher than 3% represses the production of protease. However, at low concentration (1%) it is the best alternative source of carbon after glucose for production of protease. Regarding, the nitrogen source, maximum protease production was observed when 1% yeast extract was used in GYE medium compared to other sources.

Inorganic nitrogen sources inhibited protease production and the phenomenon is termed as ammonium repression. The ratio of the concentration of carbohydrate source and the concentration of nitrogen source is kept the same as in the original GYE medium. Any other ratio affected the production of protease as seen in **Figure 8**.

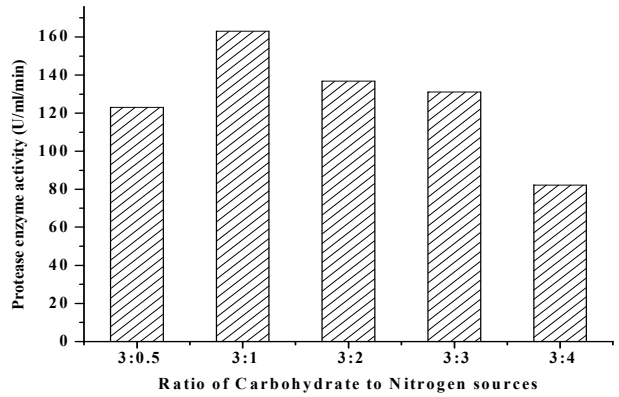


Figure 8
Effect of different ratios of Carbohydrate to Nitrogen sources (keeping constant concentration of carbon sources) on protease enzyme activity

Although the isolate could tolerate NaCl concentration upto 1%, the production of protease was adversely affected by any concentration above 0.5% NaCl (as seen in **Figure 9**), provided the isolate does not belong to halophilic group.

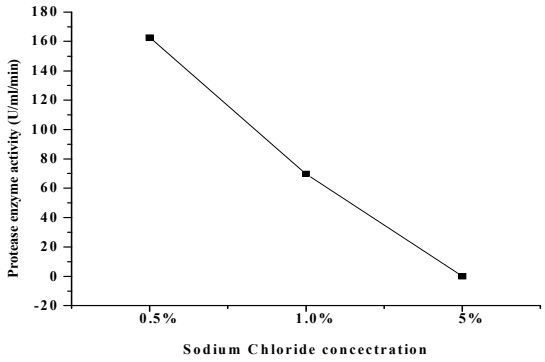


Figure 9
Tolerance to different concentrations of Sodium chloride

Almost every medium reporting the growth of Thermophilic actinomycetes, contains either $CaCO_3$ or $CaCl_2$. Among these $CaCl_2$ is required in less concentration and is easily soluble in aqueous phase compared to $CaCO_3$ as seen in **Figures 10 and 11**.

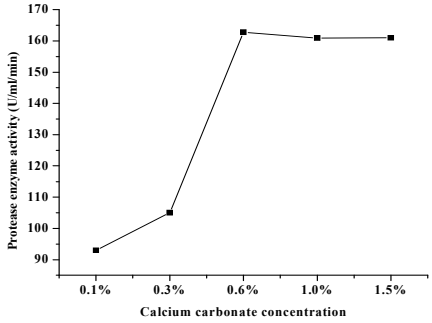


Figure 10
Effect of different concentrations of Calcium Carbonate on protease enzyme activity

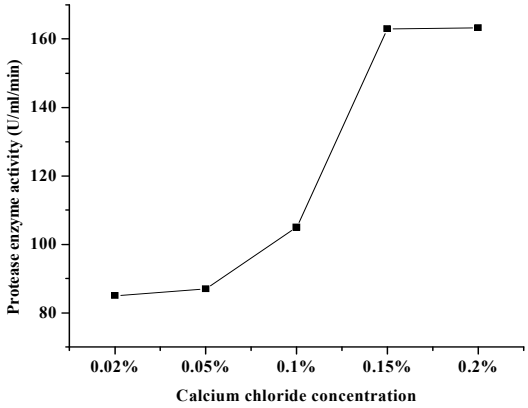


Figure 11
Effect of different calcium chloride concentration on protease enzyme activity

Figure 12 indicates the effect of addition of solutions of trace salts (K and Fe) resulting in enhanced production of the enzyme.

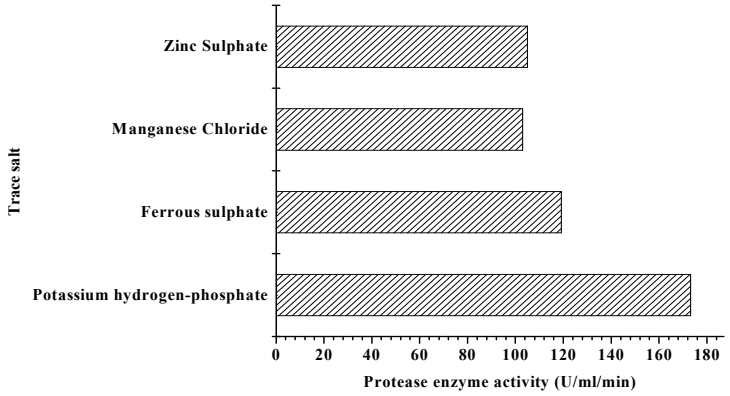


Figure 12
Effect of different trace salts on protease enzyme activity

Effect of supplements like Casamino acid (as a free amino acid) and trace salt like K_2HPO_4 have already been observed in **Figure 4** where MCY medium had both the supplement. Effects of different free amino acid other than Casamino acid, trace salts other than K_2HPO_4 and role of free amino acids at 1% and 0.5% are depicted in **Figures 13-14** respectively.

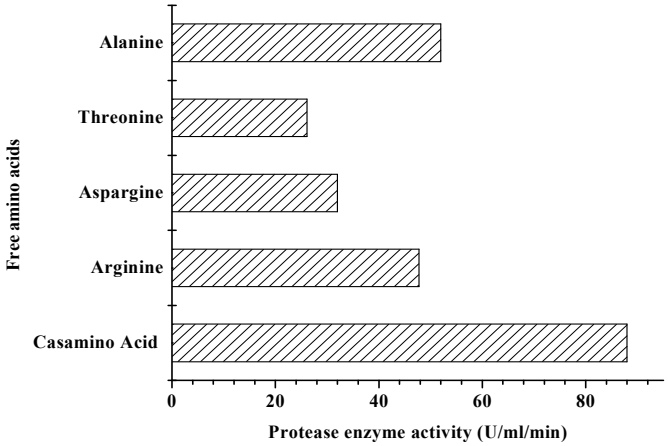


Figure 13
***Protease enzyme activity influenced by different free amino acids at 1%* concentration**

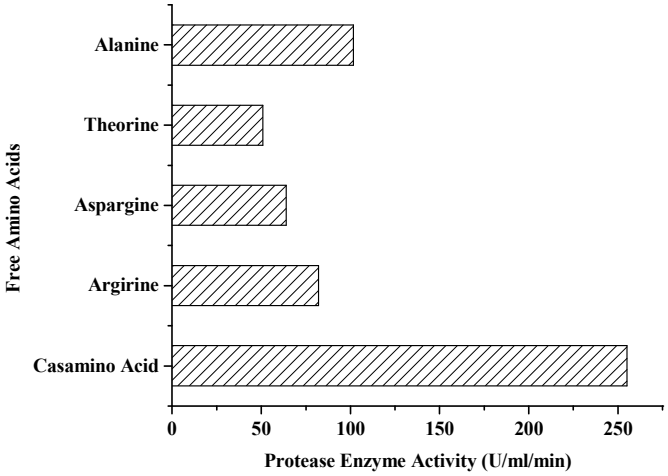


Figure 14
Protease enzyme activity influenced by different free amino acids at 0.5% concentration

Overall, Casamino acid and K_2HPO_4 had positive effect on the protease production.

Inoculum size required for optimum protease production is 0.5% if spore stock is the inoculum (**Figure 15**) while it is 1.5% if seed culture is used as the inoculum as observed in **Figure 16.**

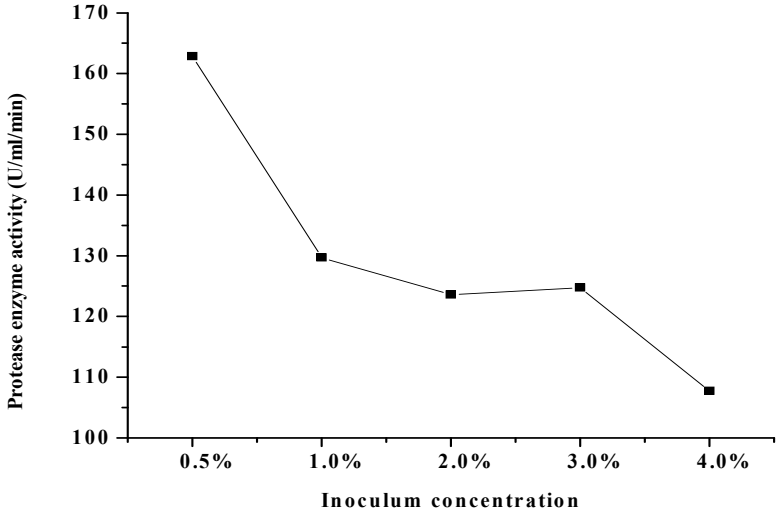


Figure 15
Inoculum size for seed culture from stock

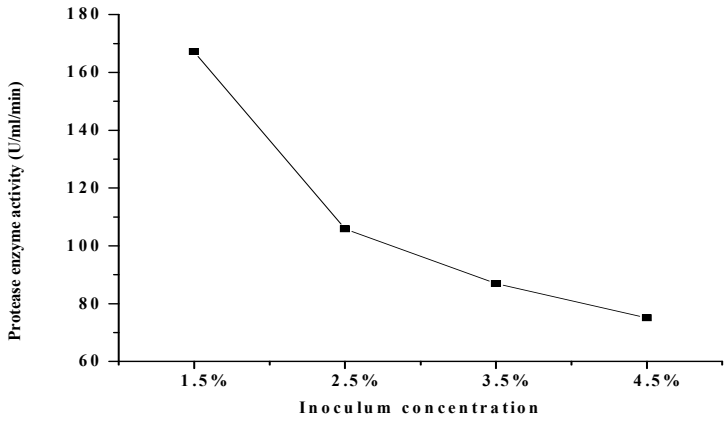


Figure 16
Inoculum size for fermentation (seed to fermenter)

Different media which were screened for protease production, had neutral pH and for all cases the incubation temperature was 55°C. The isolate PD1 is an aerobic organism which gives maximum protease production using 50 ml GYE medium taken in 250ml flask. Effect of agitation is depicted in Fig. 17. (**Figure 17)**

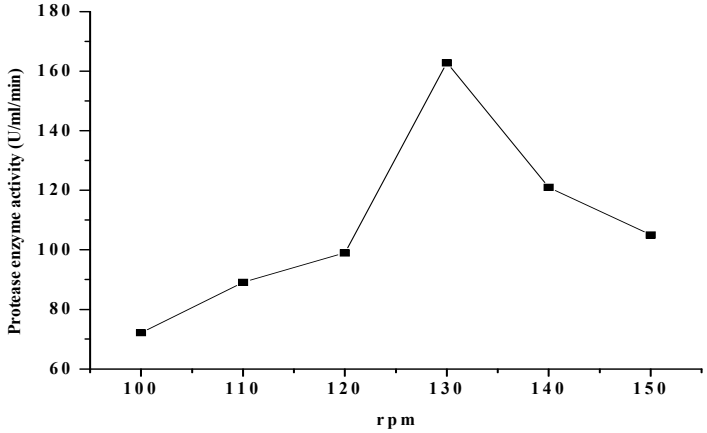


Figure 17
Agitation in 50 ml medium in 250 ml flask

DISCUSSION

The extracellular protease activity of thirty four isolates was determined qualitatively and quantitatively. The activities exhibited by all thirty four isolates in liquid and solid media were compared and there was a definite correlation between protease activity and the clear zone on CGYP agar as reported earlier[2].

Extracellular protease activity from the isolate. PD1

The white precipitate identified as aspartame with the help of HPLC[25] was identical with the aspartame production by thermolysin from *Bacillus thermoproteolyticus.* (Rokko)[23]. The isolate identified with the help of various morphological, biochemical and molecular biology viz. 16sRNA techniques was

Thermoactinomyces thalpophilus[14, 19, 20] . The production of protease with relation to growth of PD1, measured in terms of total protein content of biomass is a typical characteristic of genus *Thermoactinomyces* as described by Lacey previously[14].

Production of protease enzyme determined in different media and yield of the enzyme in terms of activity was maximum (265 U/ml/min) in MCY medium which had Casamino acid[13, 14]. These authors have reported the effect of 0.5% of Casamino acid on protease enzyme production. According to literature reports glucose seems to be the most preferred source of carbohydrate for the growth and production of protease enzyme. Maltose at low concentration (1%) is the best alternative source of carbon after glucose for production of protease. Inorganic nitrogen sources inhibited protease production as reported earlier (Frankeva *et al.* 1986) and the phenomenon is termed as ammonium repression. The ratio of the concentration of carbohydrate source and the concentration of nitrogen source is kept the same as in the original GYE medium as reported in literature[3]. Almost every medium reporting the growth of thermophilic actinomycetes, contain either $CaCO_3$ or $CaCl_2$. Dependence of thermophilic actinomycetes on calcium salts is reported by several workers[1, 3, 12, 14, 18, 21]. Iron in low concentration acts as an inducer as previously reported[2].

Overall, Casamino acid, K_2HPO_4 and sodium perborate had positive effect on the protease production which is in agreement with previous literature reports[2,14,18].

Different media which were screened for protease production, had neutral pH and for all cases the incubation temperature was 55°C which is in agreement with literature reports[14]. The isolate PD1 appears to be an aerobic organism which gives maximum protease production using 50 ml GYE medium taken in 250ml flask as described[14].

CONCLUSION

Present work thus describes successful screening and identification of the isolate *Thermoactinomyces thalpophilus* producing the TLP enzyme which is capable of producing artificial sweetening agent, viz. aspartame, as determined by HPLC. The optimization of physical, chemical and biological parameters has been carried out to maximize the yield of protease enzyme in terms of activity.

***Abbreviation*:** TLP, thermolysin like protease; GYP, glucose yeast extract peptone; CGYP, Casein glucose yeast extract peptone ; TCA, Trichloroacetic acid; BSA, bovine serum albumin; TFA, Trifluoroacetic acid; CFB, Cell free broth; SDS, Sodium dodecyl sulfate

REFERENCES

1. Allen, M.B., The thermophilic aerobic spore-forming bacteria. Bacterological Reviews 17: 125-173, (1953)
2. Chaphalkar, S.R., Dey, S., Some aspects of production of extracellular protease from *Streptomyces diastaticus*. J Microbiol. Biotech 9: 85-100, (1994)
3. Chitte, R.R., Nalawade, V.K., Dey, S., Keratinocytes activity from the broth of a feather degrading thermophilic *Streptomyces thermoviolaceus* strain SD8. Letters in Applied Microbiology 28: 131-136, (1999)
4. Chitte, R.R., Dey, S., Potent fibrinolytic enzyme from a thermophilic *Streptomyces megasporus* strain SD5. Letters in Applied Microbiology 31: 405-410, (2000)
5. Yallop, C.A., Edwards. C., Williams, S.T., Isolation and growth physiology of novel

Thermoactinomycetes. Journal of Applied Microbiology 83: 685-692, (1997)
6. Brock, T.D., Thermophiles. General, molecular and Applied Microbiology, John Wiley and Sons, New York: 1-16, (1986)
7. Matsubara, H., Purification and assay of thermolysin methods in Enzymology 19: 642-651, (1970)
8. Kuhn, D., Durrschmidt, P., Mansfeld, J., Ulbrich Hofmann, R. Biolysin and Thermolysin in dipeptide synthesis: a comparative study. Biotechnology and Applied Biochemistry 36: 71-76, (2002)
9. Durrschmidt, P., Manofeld, J., Ulbrich-Hoffman, R., Differentiation between conformational and autoproteolytic stability of the neutral protease from *Bacillus stearothermophilus* containing an engineered disulfide bond. Eur J Biochem 268: 3612-3618, (2001)
10. Odibo, F.J.C., Obi, S.K.C., Purification and some properties of a thermostable protease of *Thermoactinomyces thalpophilus*. World Journal of Microbiology and Biotechnology 4: 327-332, (1988)
11. Georgieva, D.N., Stoeva, S., Ivanova, V., Gusterova, A., Voelter, W., Specificity of a neutral Zn-dependent proteinase from *Thermoactinomyces sacchari* toward the oxidized insulin B chain. Current Microbiology 41: 70-72, (2000)
12. Zabolotskaya, M,V., Demidyuk, I.V., Akimkina, T.V., Kostrov, S.V. A Novel Neutral Protease from *Thermoactinomyces* species 27a: Sequencing of the gene, purification, and characterization of the enzyme. The Protein Journal 23, 483- 492, (2004)
13. Tsuchiya, K., Sakashita, H., Nakamura, Y., Kimura, T., Note-Production of Thermostable Alkaline Protease by Alkalophile. *Thermoactionomyes* Sp. HS 682. Agric. Biol. Chem. 55: 3125-3127, (1991)
14. Lacey, J., In section 32, Actinomycetes. Bergey's Manual of systematic bacteriology. Vol 4. Williams and Wilkim Publication (USA). 2573-2585, (1989)
15. Burgess, R.R., Arthur, T.M., Pietz, B.C., Mapping Protein-Protein Interaction Domains using ordered fragment ladder far-western analysis of His_6 – Tagged protein fusions. Methods in enzymology 328: 141-157, (2000)
16. Dunn, B.M., Determination of protease mechanism. In: Beynon, R.J., and Bond, J.S. (eds.). Proteolytic enzyme: a practical approach. (Oxford: IRL Press): 57-82, (1996)
17. Hase, C.C., Finkelstein, R.A., Bacterial extracellular zinc – containing metalloproteases. Microbiological Reviews. American Society for Microbiology. 54: 823-837, (1993)
18. Takami, H., Akiba, T., Horikoshi, K., Production of extremely thermostable alkaline protease from *Bacillus* sp.no.AH-101. Applied Microbiology and Biotechnology 30: 120-124, (1989)
19. Chakraborty, T., Actinomycetes – Laboratory Manual. MTCC. IMT. Chandigarh India, (1998)
20. Goodfellow, M., Mordarski, M., Williams, S.T., The Biology of the Actinomycetes. Academic Press Inc. (London). Ltd.: 481-513, (1984)
21. Fujii, M., Takagi, M., Imanaka, T., Aiba, S. Molecular cloning of a thermostable neutral protease gene from *Bacillus stearothermophilus* in a vector plasmid and its expression in *Bacillus stearothermophilus* and *Bacillus subtilis*. Journal of Bacteriology 154: 831-837, (1983)
22. Lowry, O.H., Rosenbrough Farr, A.L., Rondall, R.J., Protein measurement with folin phenol reagent. J. Biol. Chem. 193: 265-275, (1951)
23. Isowa, Y., Ohmori, M., Ichikawa, T., Mori, K., Nonaka, Y., Kihra, K., Oyama, K.,

Satoh, H., Nishimura, S. Thermolysin-catalysed condensation reactions of N-substituted aspartic and glutamic acids with phenylalanine alkyl esters. Tetrahedron Letters. 28: 2611-2612, (1979)

24. Mazur, R.H., Schlatter, J.M., Goldkamp, A.H., Structure – Taste relationships of some dipeptidases. Journal of the American Chemical Society, 91: 2684-2691, (1969)
25. Neumeister, C., Aspartame Method 5031, Issue 2, NIOSH. Manual of Analytical Methods (NMAM). 4th edn., (1994)