CHAPTER IV

EFFECT OF NITROGEN SOURCE ON PRODUCTION OF MILK CLOTTING ENZYME OF SELECTED LAB

4.1 INTRODUCTION

Factors such as bacterial cultures, medium, composition and cultivation condition (pH, temperature of incubation) and speed of shaker influence the MCE production (Zhang et al., 2011; Darah & Lim, 2013). Earlier reports in *Bacillus* spp. indicated that different nitrogen sources such as soybean meal, casamino acid, and peptone were effective medium ingredients for the proteinase production (Mehrotra et al.1999; Joo et al. 2002; Puri et al. 2002; Joo & Chang 2005; Patel et al. 2005; Chu, 2007). The extracellular proteinase production of *B. horikoshii* was also influenced by concentration of nitrogen as reported by (Joo et al., 2002). Naidu and Devi, (2005) reported that the suitable nitrogen source for proteinase production of *Bacillus* sp. K30 was beef extract, yeast extract, tryptone and peptone.

Few studies reported the effects of nitrogen sources on the production of extracellular proteinase by LAB (Sato et al., 2004). Herbert et al. (2001) observed that *L. helveticus* grew and produce highest MCE in 2% of yeast, 5% casein and 1% sodium caseinate. Sato et al. (2004) added to MRS broth different nitrogen sources trypticase pepton and casein to screen extracellular proteinase production by *E. faecalis* 2495L. Similarly, Akinkugbe and Onilude (2013); Oke and Onilude (2014) observed that MRS broth supplemented with 2% casein was suitable as nitrogen source for the enzyme

production by *L. acidophilus* and *P. acidilactici*. Casein when added to enzyme production media was reported to enhance the production of extra cellular proteinase by marine *Pseudomonas* strain 1452 (Nigam et al., 1981). Casein proved to be an excellent substrate for the production of the milk-clotting enzyme from *M. mucedo* DSM 809 (Yegin et al., 2010).

Methods that commonly used for partial purification of the culture supernatant are ammonium sulphate and solvent precipitation (Tunga et al., 2003; Fernandez et al., 1999). Generally, precipitation of proteins using ammonium sulfate was used as the first step in the enzyme purification (Anil & Neha, 2006). Ammonium sulphate was used because of its high solubility, salting out effectiveness, pH versatility and low heat of solution, cheaper and stabilizing effect on most enzymes (Lee et al., 2002; Cheng et al., 2010). However gel filtration chromatography is one of the techniques that separate the proteins based on the size and shape. Columns of gel filtration are made of porous bead; can be polymers of dextran (Sephadex), agarose (Sepharose and Superose), agarose cross-linked to dextran (Superdex), polyacrylamide (Sephacryl) (Richardson et al., 1967). Each bead has different physical properties that may make them more appropriate for different proteins and also can be used as a desalting technique (Andrews, 1965). Sephadex G-50 with sodium phosphate buffer 50 mmol were used for the purification of extracellular proteinase produced by *Micrococcus* (Fernandez et al., 1996), *E. faecalis* subsp (De-Fernando et al., 1991) and extracellular proteinases from *Coprinus cinereus*

(Kalisz et al., 1989). Alkaline protease produced by *Microbacterium* sp. strain KR10 was purified by sequential liquid chromatography on Sephadex G-100.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is an excellent method with which to identify and monitor proteins. The most widely used method was developed by Laemmli, (1970) using the denaturing (SDS) discontinuous method. This procedure depended on the presence of SDS (sodium dodecyl sulfate) and ß-mercaptoethanol to denature the proteins using different buffers of pH. Wu et al. (2013) observed two major bands 25 and 30 KDa present in MCE from *B. natto* using SDS-PAGE 12.5%. MCE produced by *E. faecalis* TUA2495L was 34 KDa (Sato et al., 2004); MCE produced by *L. casei*- D1-1 was 35 KDa (Xing et al., 2012). Purified MCE from *B. subtilis* K-26 showed a molecular mass of 27 KDa (Rao et al., 1979). The aim of the present study was to determine the effect of the nitrogen source on MCE of *P .acidlactici* SH, and *L. paracasei* CF1. Partially purification of the enzyme produced was carried out using different concentration of ammonium sulphate, followed by gel filtration column using sephadex G-50 fine coloum. The MCA and ratio of MCA/PA were calculated to determine of the enzyme is suitable in dairy products.

4.2 MATERIALS AND METHODS

4.2.1 Effect of Nitrogen Sources on the Production of Milk Clotting Enzyme

The effect of nitrogen source concentration on MCA was determined according to Dutt et al. (2008). The enzyme production media preparation and incubation conditions were

similar to that described in Chapter III section 3.3.8 except that different nitrogen sources were used namely, tryptone soya, casein, tryptophan and trypticase peptone at varying concentrations (0.5%, 1%, 1.5%, 2%, 2.5% w/v). MCA and PA were determined as described in Chapter 3 3.2.

4.2.2 Determination of Protein Concentration

Total protein concentration of samples was determined by Bradford method using bovine serum albumin (BSA, 0.2 mg/ml) as the standard (Bradford, 1976). BSA standards were prepared in different concentrations and the standard calibration curve was drawn with response to their absorbance values. Total protein content was calculated from standard calibration curve equation. The changing of the absorbance was recorded spectrophotometrically at 595 nm in UV-visible spectrophotometer. The blank includes 800 µl water and 200 µl Bradford reagent. The sample included 2 µl protease enzyme, 798 µl water and 200 µl Bradford reagent. The solutions were kept at room temperature for 5 minutes followed by the addition of Bradford reagent. The changing of the protein concentration of the sample was determined at 595 nm. The spectrophotometric assays were performed using UV-VIS spectrophotometer with a constant temperature water circulator.

4.2.3 Partial Purification of Milk Clotting Enzyme

4.2.3.1 Ammonium Sulphate Precipitate

Solid ammonium sulphate (20 to 80% saturation) was added to a chilled crude enzyme preparation to precipitate the enzyme and was carried out at 4°C. The precipitate obtained was collected by centrifuge at 8000 × g and 4°C for 15 min, (COMBI 415 R HANIL SCIENC INDUSTRIAL), and dissolved in a minimum quantity of Tris–HCL buffer pH 8.5. This partially purified enzyme was dialyzed using dialysis tubing with molecular cut-off 12 000 to 14 000 Da. MCA and protein content were determined for each fraction.

4.2.3.2 Gel Filtration Using Sephadex G-50 Fine

Fractions of ammonium sulfate with high MCA was lyophilized and then applied to Sephadex G-50 fine (GE. HEALTHCARE LIFE SCIENCES) column (1.5 × 55 cm) previously equilibrated with 0.2 M phosphate buffer pH 7.0. The enzyme was eluted with the same buffer at a flow rate of 0.6 mL/min. MCA and protein content were determined in each fraction. The fractions that showed MCA were collected and concentrated by lyophilization.

4.2.4 Preparation of Sodium Phosphate Buffer

Sodium phosphate (0.2 M) pH 7 was prepared as follows: Solution A was prepared by dissolving 13.8 g of monobasic sodium phosphate (M.W137.99 g/mol) in 500 ml

deionized water in volumetric flask. Solution B was prepared by dissolving 17.8 g of sodium phosphate dibasic (M.W. 177.99 g/mol) in 500 ml deionized water in volumetric flask. Solution A (39 mL) was mixed with solution B (61 mL) and bring up the volume with deionized water in 100 mL volumetric flask.

4.2.5 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE)

The molecular weight of the purified enzyme was estimated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) as previously described by (Laemmli, 1970).

4.2.6 Preparation of 4% Stacking Gel

The stacking gel was prepared following the formula as shown in Table 7 as described by Laemmli (1970).

Table 7: Stacking Gel Solution (4% Acrylamide)

Ingredients	Volume (ml)
H_2O	3.075
0.5 M Tris-HCl, pH 6.8	1.250
20% (w/v) SDS	0.670
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	0.670
10% (w/v) ammonium persulfate (APS)	0.025
TEMED	0.005

4.2.6 Preparation of Running Gel Solution (12.5%)

Preparation of the running gel solution was as described in the Table 8. Dissolve the mixture completely, and then set the casting frames (clamp two glass plates in the casting frames) on the casting stands. Then pipette appropriate amount of the running gel solution into the gap between the glass plates. Then wait for 15 to 20 min to let it solidify.

Table 8: Running Gel Solution (12.5%)

Ingredients	Volume (ml)
H_2O	10.2
0.5 M Tris-HCl, pH 8.8	07.5
20% (w/v) SDS	0.15
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	12.0
10% (w/v) ammonium persulfate (APS)	0.15
TEMED	0.02

4.2.7 Preparation of Sample Buffer

Stock sample buffer (0.06 M Tris-HC1, pH 6.8, 2% SDS, 10% glycerol, 0.025% Bromphenol Blue) was prepared as shown below:

Table 9: Preparation of the Sample Buffer

Ingredients	Volume (ml)
H_2O	4.80
0.5 M Tris-HCl, pH 6.8	1.20
10% (w/v) SDS	2.00
GIycerol	1.00
0.5% Bromphenol Blue (w/v water)	0.50

4.2.8 Preparation of the Running Buffer

To prepare one liter of running buffer 0.025 M Tris, 0.192 M glycine, 0.1% (w/v) SDS, pH 8.3).solution of 3 g Tris base, 14 g glycine, 10 ml 10% SDS in 1000 ml of distilled water running buffer. No need to adjust the pH of the running buffer; just mix the reagents together and confirm that the pH is near 8.3 (± 0.2).

4.2.9 Preparation of Partially Purified Enzyme Fractions for SDS PAGE

The fractions after gel filtration that showed higher MCA was mixed 1:1 with sample buffer as described in section 4.4.8 then heated to 95°C in a water bath for 5 min followed by cooling to room temperature approximately 25°C. Stacking gel (4% acrylamide) and running gel (12.5% were prepared as described above. Treated enzyme samples 20ul were loaded into the well of the stacking gel and electrophoresis was carried out at constant current 120 Volts for 120 min. The gel was carefully removed from between the glass plates and put in the fixing solution consists of 40% of methanol mix with 10% of acetic acid (v/v) for 30 min. The gel was stained for 3 h with 0.1% of Coomassie Blue R250 staining (BIO BASIC CANADA INC.) in 40% of methanol (v/v) and 10% of acetic acid (v/v). The gel was destained using destaining solution consisting of (4: 5: 1) (v/v/v) methanol, water and acetic acid. The molecular weight of the MCA was determined by using standard molecular weight markers 10 to 250 KDa (Bio Rad laboratories, Inc. BIO-RAD).

4.2.10 Statistical Analysis

A two way analysis of variance (ANOVA) using MINITAB version 16 was carried out at ($P<0.05$) for significant differences. Tukey's Pairwise Comparison was used to compare mean differences on the effects of nitrogen sources and concentrations and their interactions on MCA.

4.4 RESULTS

4.4.1 Effect of Nitrogen Sources on Production Milk Clotting Enzyme of Selected Lactic Acid Bacteria

Among the nitrogen sources used (casein, peptone soya, tryptophan and trypticase peptone) to produce MCE by *P. acidlactici* SH, and *L. paracasei* CF1 at different concentration (0.5% to 2.5%), maximum MCA was observed when 0.5% casein was used in the enzyme production media while the tryptophan showed lowest MCA. The values of MCA, PA and MCA/PA of isolates *P. acidlactici* SH, and *L. pracasei* CF1 were significantly ($p<0.05$) affected by type of nitrogen sources and the concentration of the nitrogen sources. Since casein showed to be an excellent substrate for the production of the MCE by *P. acidlactici* SH and *L. paracasei* CF1, casein was selected as a nitrogen source in the enzyme production media. Culturing of *P. acidilactici* SH in enzyme production media containing 0.5% casein as nitrogen source resulted in maximum MCA (75 SU/ml), high ratio of MCA/PA (37.5) and low PA (1.9 U/ml). These values were significantly different ($P<0.05$) from culturing *P. acidilactici* SH in other nitrogen sources (Figures 7, 8 and 9). MCE was not produced

at 2.5% casein. Culturing *L. paracasei* CF1 in enzyme production media containing 0.5% casein as nitrogen source produced MCA of 54 SU/ml, (Figure 10) lower than *P. acidilactici* SH with PA value of 2.6 U/ml (Figure 11). However, production of MCE decreased at higher casein concentration (>1.5%) after 48 h at 30ºC of cultivation. In contrast the highest MCA (57 SU/ml) and MCA/PA ratio (21.1) were observed using 1% casein as a nitrogen source in enzyme production media (Figure 12).

Figure 7: Effect of Organic Nitrogen Sources on MCA using MCE from *P. acidilactici* SH

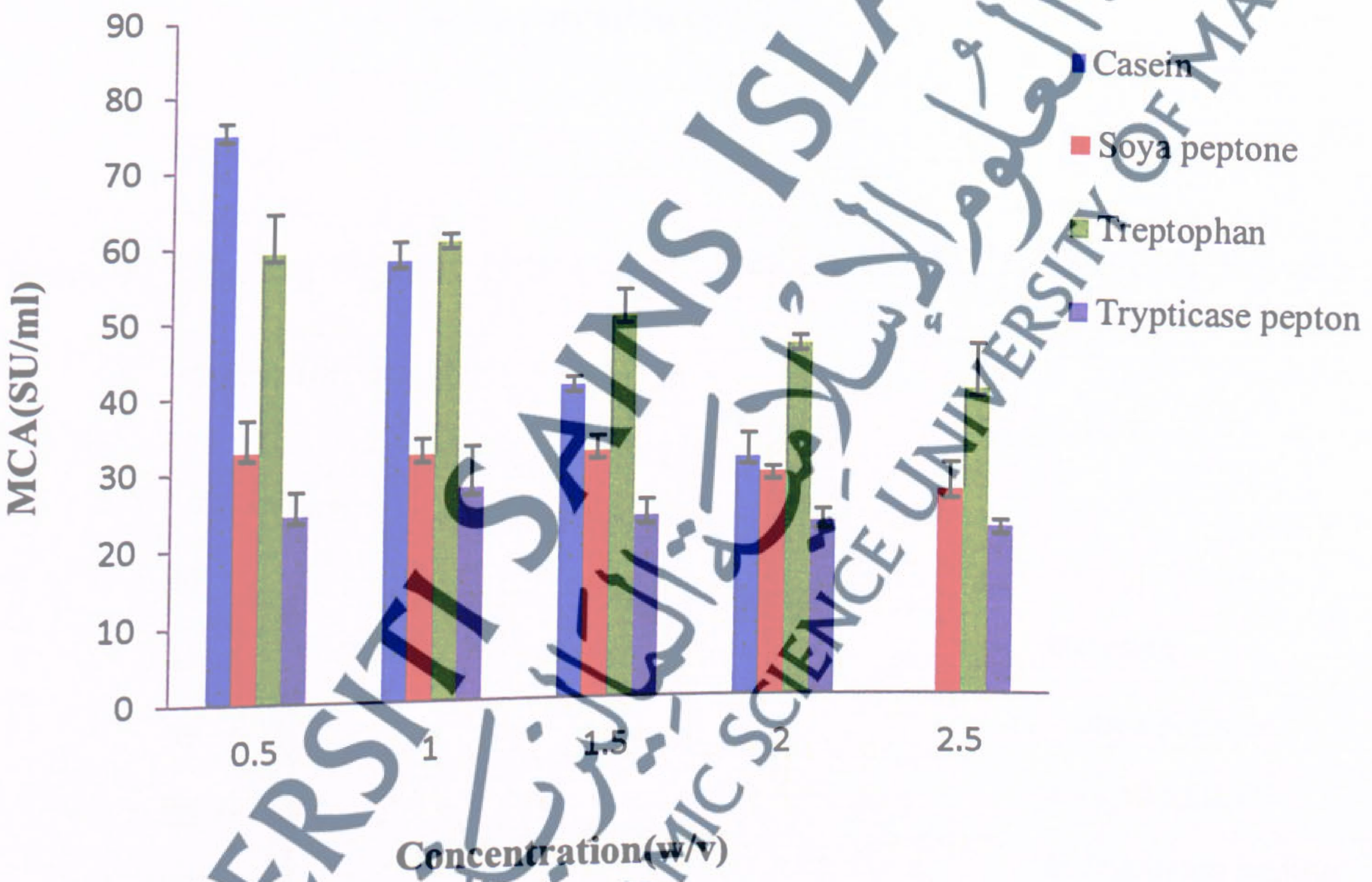


Figure 8: Effect of Organic Nitrogen Sources on PA using MCE from *P. acidilactici* SH

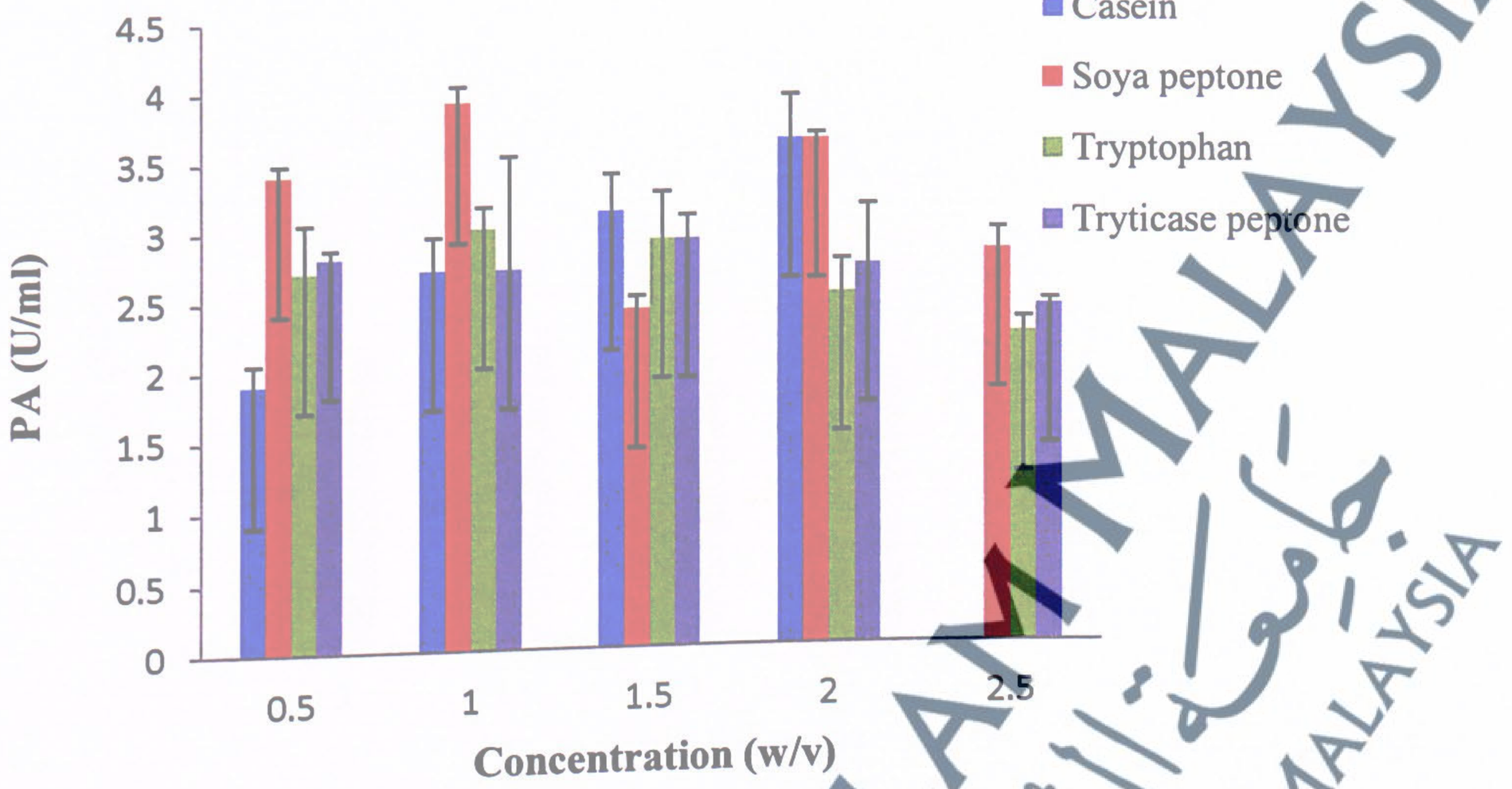


Figure 9: Effect of Organic Nitrogen Sources on MCA/ PA using MCE from *P. acidilactici* SH

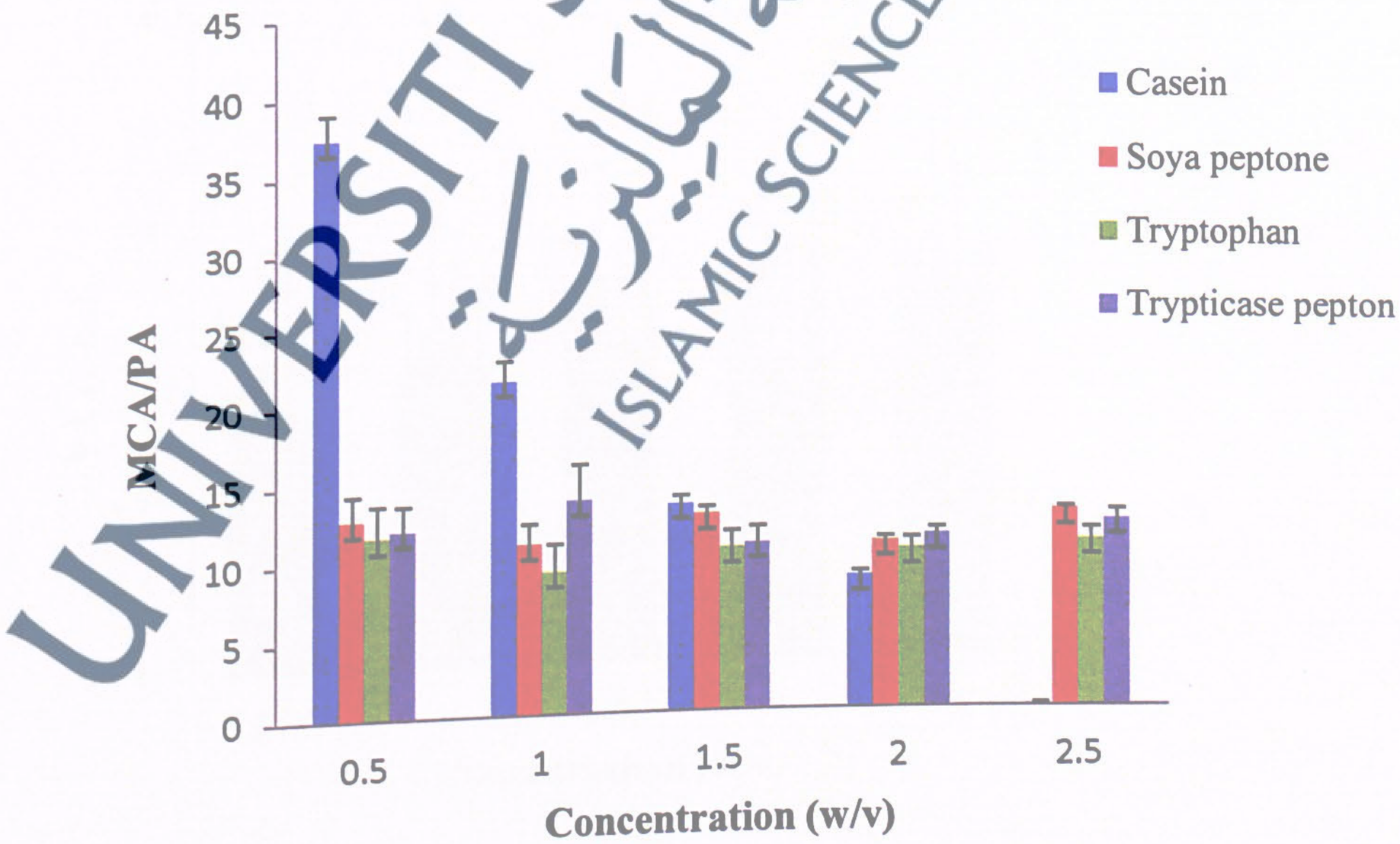


Figure 10: Effect of Organic Nitrogen Nources on MCA using MCE from *L. paracasei* CF1

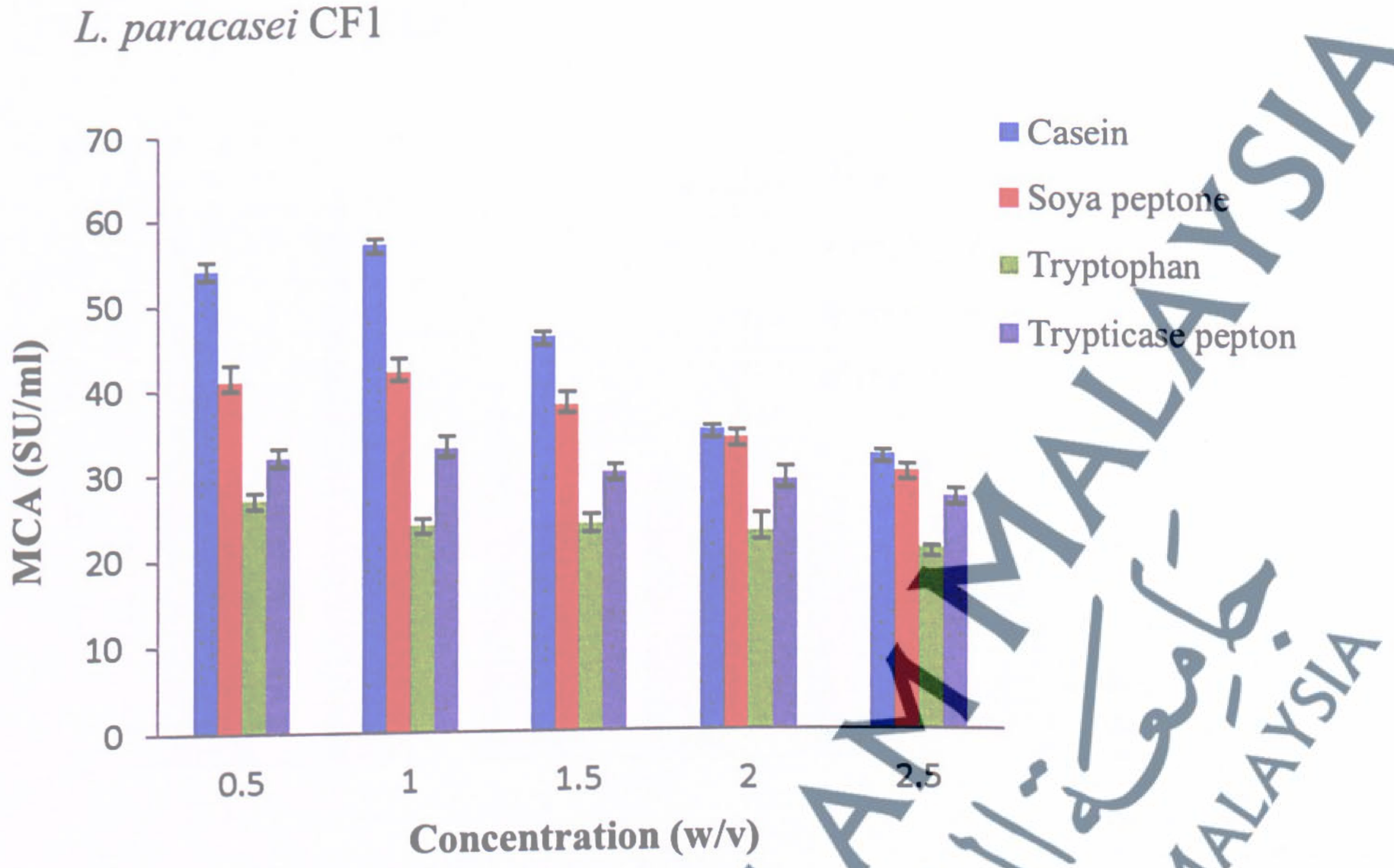


Figure 11: Effect of Organic Nitrogen Sources on PA using MCE from *L. paracasei* CF1

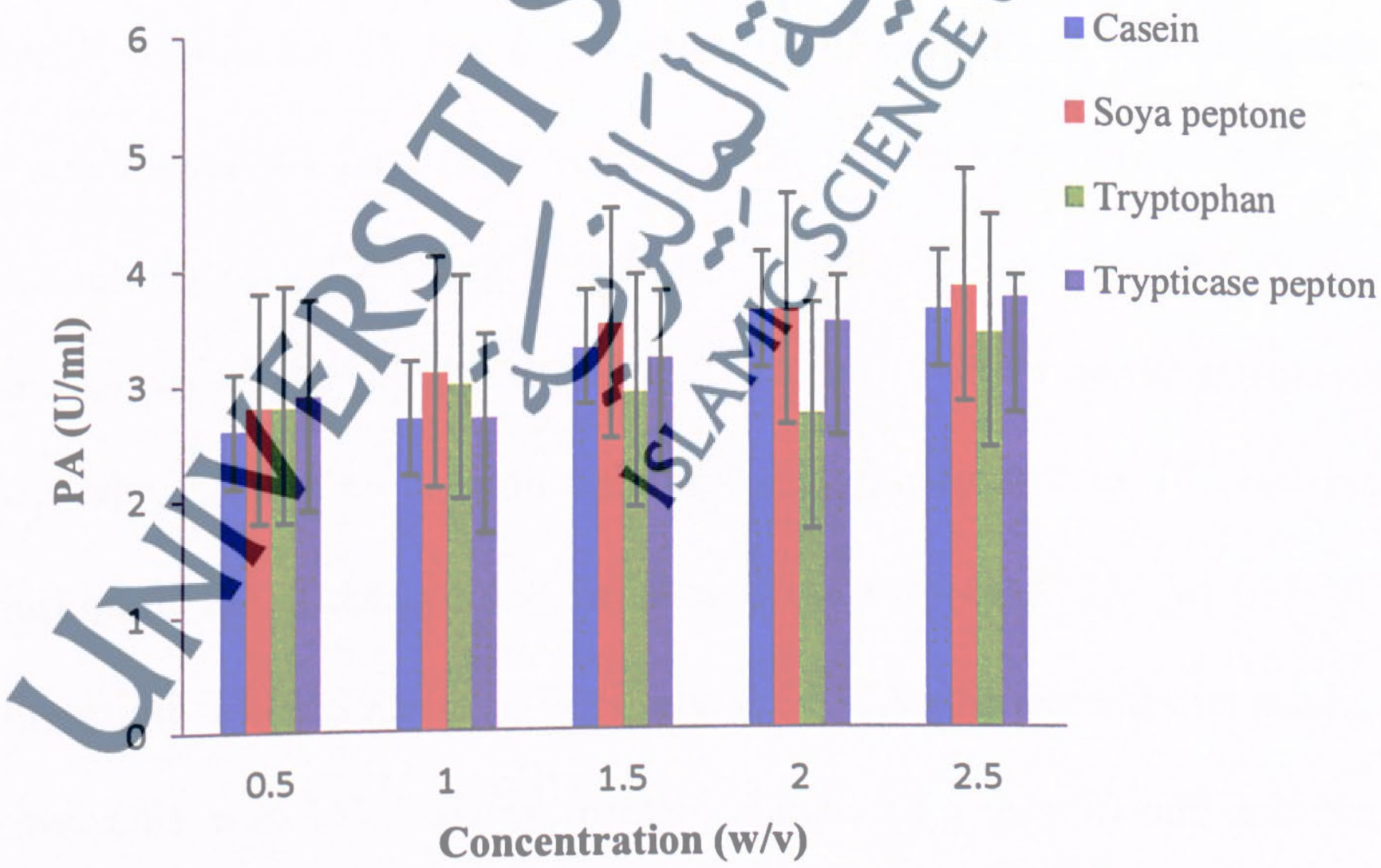
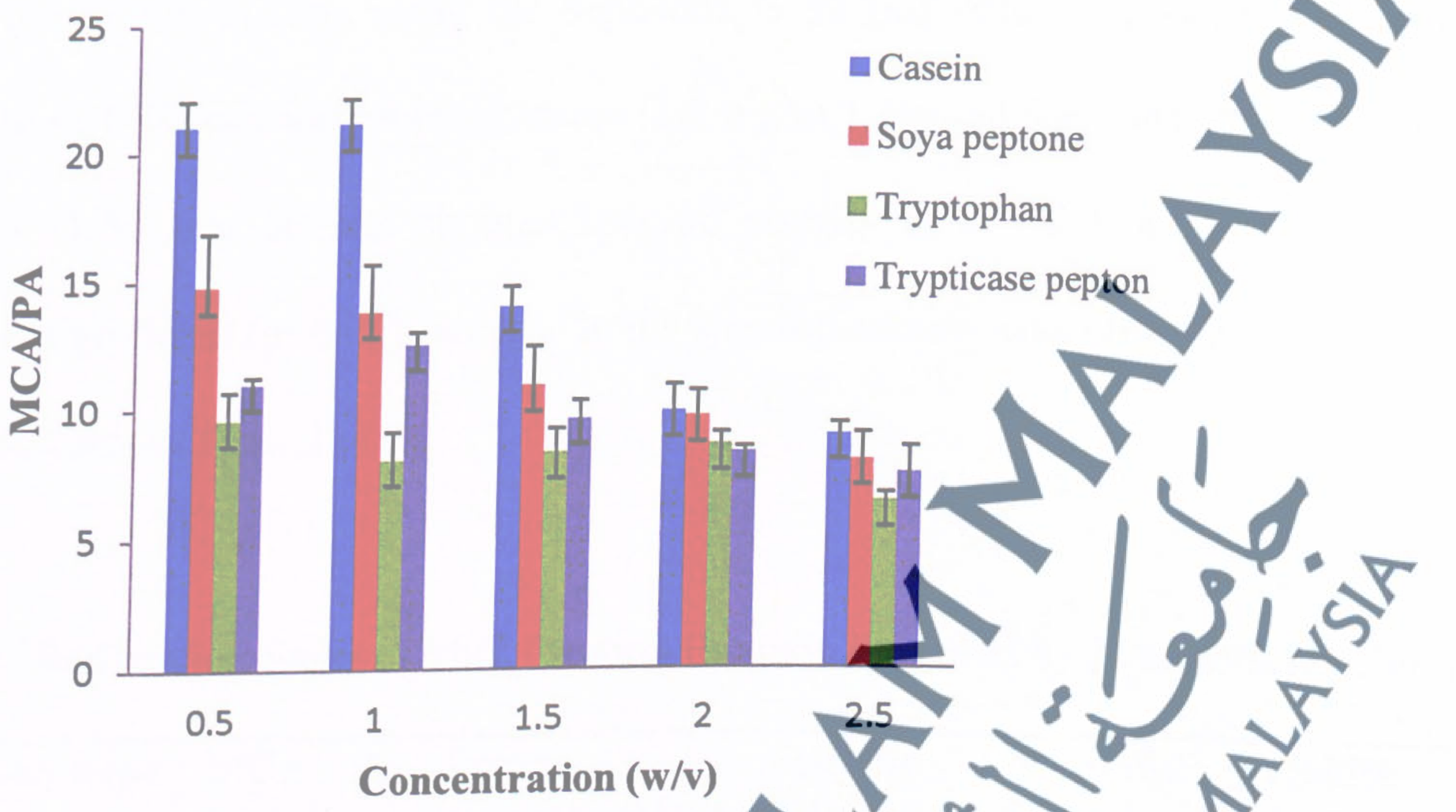


Figure 12: Effect of Organic Nitrogen Sources on MCA/ PA f using MCE *L. paracasei* CF1



4.4.2 Purification of Milk Clotting Enzyme by Ammonium Sulphate Precipitation and Gel Filtration

Partial purification of crude MCE using 60% ammonium sulphate showed high MCA for both *P. acidilactici* SH and *L. paracasei* CF1 (Table 10 & 11). The crude enzyme of *P. acidilactici* SH has MCA value 750 SU/ml, protein content 6.5 mg/ml, and specific activity of 115.4 U/mg. However, partial purification with 60% ammonium sulphate decreased slightly the MCA to 727 SU/ml). Further partial purification using the Sephadex G-50 fine column showed that fractions 7 to 14 contained high protein(Figure 13). These pooled fractions were showed MCA of 632 SU/ml and specific activity of (258.8 u/mg). Similarly, the MCA of crude enzyme produced by *L. paracasei* CF1 was 545.5 SU/ml, protein content (4.5 mg/ml) and specific activity 121.1 U/mg. Partial purification using 60% ammonium sulphate the MCA was 484

SU/ml, and protein content 3.7 mg/ml. The specific activity of partially purified MCE by 60% ammonium sulphate for *L. paracasei* recorded a value of (130.8 U/mg). Further partial purification using the Sephadex G-50 fine column reduced the MCA slightly to 421 SU/ml, and protein content (1.8 mg/ml). Pooled fractions 10 to 17 from Sephadex G-50 fine column showed specific activity of 233.8 U/mg (Figure 14). However, significant ($p<0.05$) increase in the specific activity was observed at various purification steps (Table 11).

Table 10: Purification Steps of Milk Clotting Enzyme Produced by *P. acidilactici* SH

Purification steps	MCA (su/ml)	Protein content(mg/ml)	Specific activity(u/mg)	Fold	Yield%
Crude enzyme	750±50	6.5±1.0	115.40	1.00	100
60% Ammonium sulphate	727±60	4.7±0.5	145.60	1.40	97.0
Sephadex G-50 fine	632±40	2.5±0.5	258.80[4]	2.30	84.0

Figure 13: Elution Profile of *P. acidilactici* SH MCE using Sephadex G-50 Fine using Phosphate Buffer pH 7

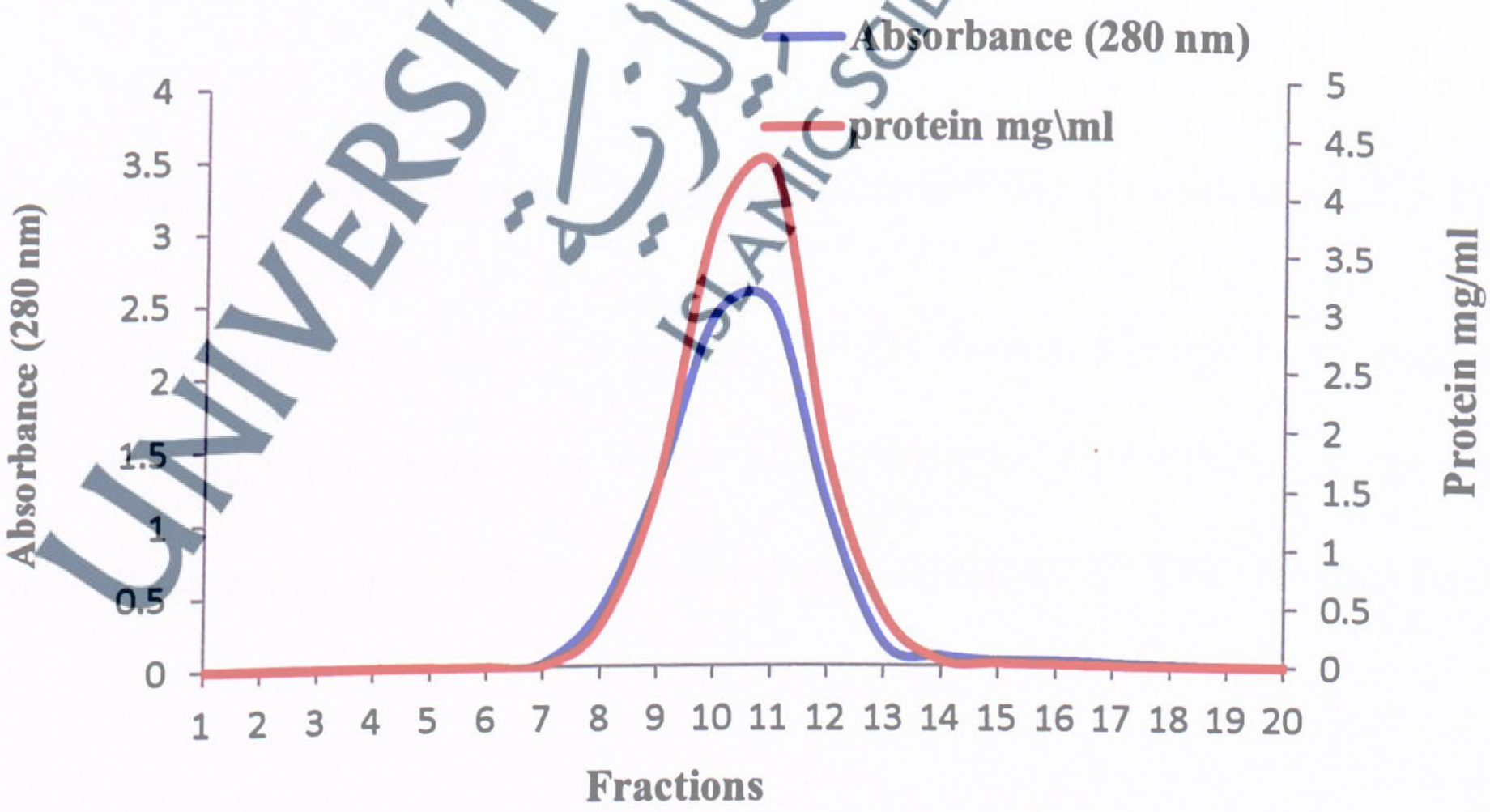
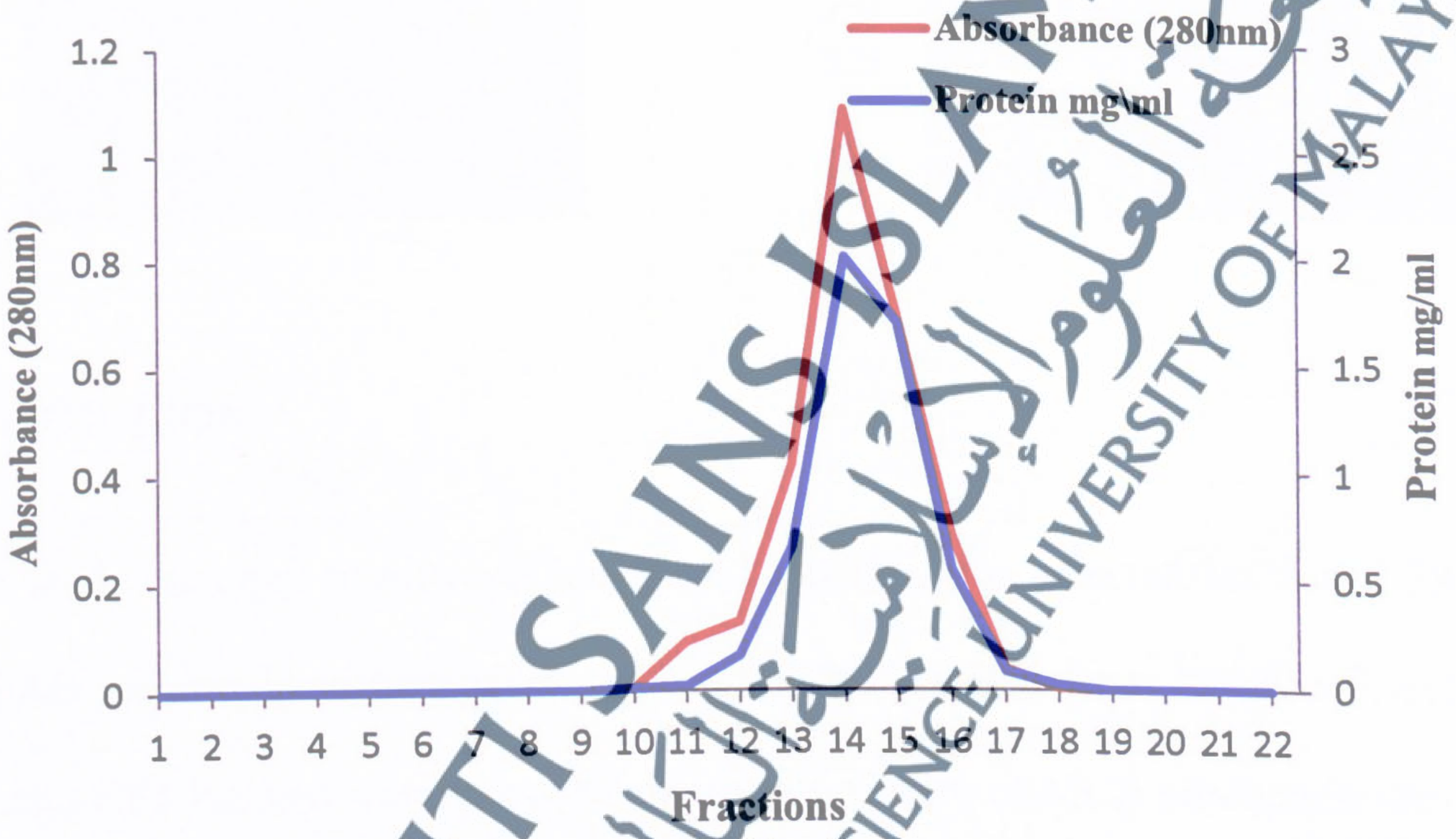


Table 11: Purification Steps of Milk Clotting Enzyme produced by *L. paracasei* CF1

Purification steps	MCA (su/ml)	Protein content(mg\ml)	Specific activity(u/mg)	Fold	Yield%
Crude enzyme	545±50	4.5±1.0	121.10	1.00	100
60%Ammonium sulphat	484±60	3.7±0.5	130.80	1.08	88.0
Sephadex G-50 fine	421±40	1.8±0.2	233.80	1.93	77.0

Figure 14: Elution Profile of *L. paracasei* CF1 MCE using Sephadex G-50 Fine using Phosphate Buffer pH 7



4.4.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE)

The partially purified enzyme of *P. acidilactici* SH showed a single band using 12.5% SDS-PAGE gel, with a molecular weight of approximately 30 KDa while the partially purified enzyme of *L. paracasei* CF1 shown approximately 27 KDa (Figure 16 & 17).

Figure 15: SDS12.5% PAGE of the Purified Enzyme from *P. acidilactici* SH

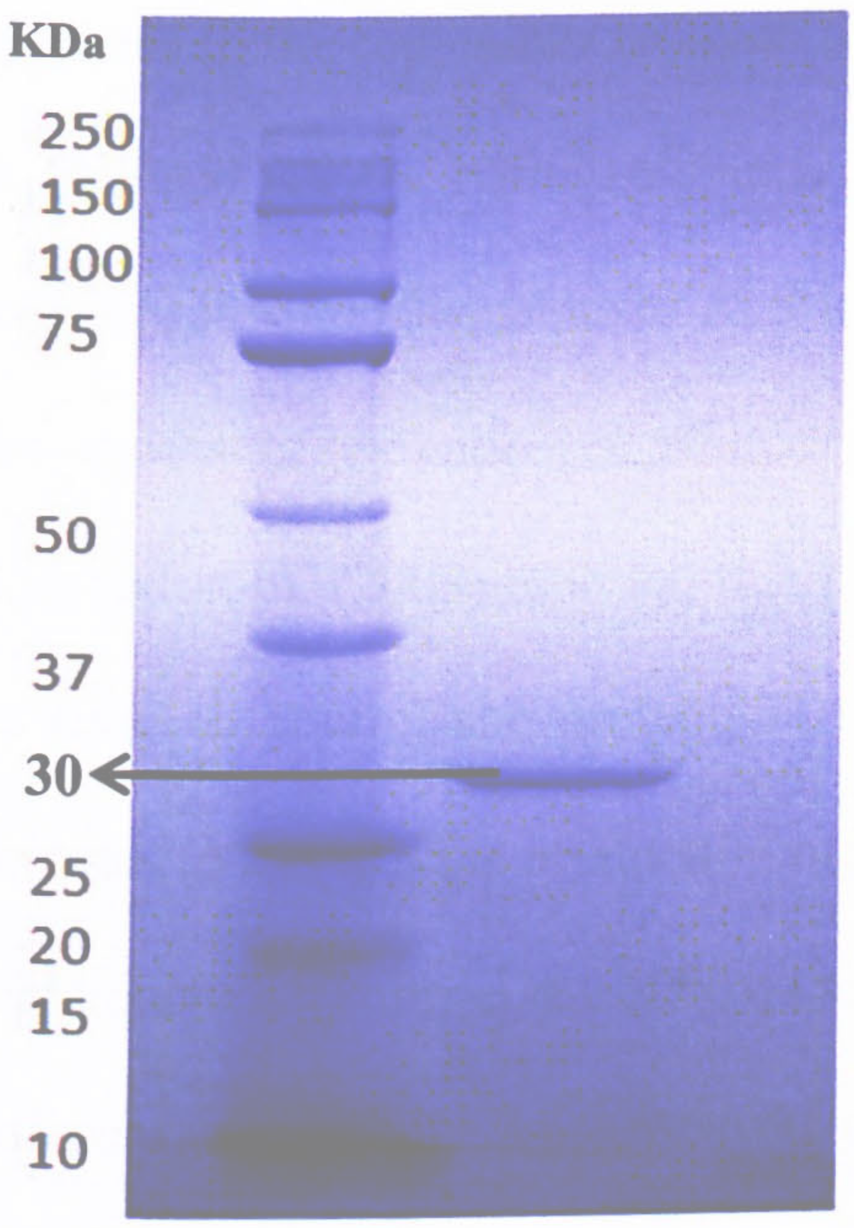
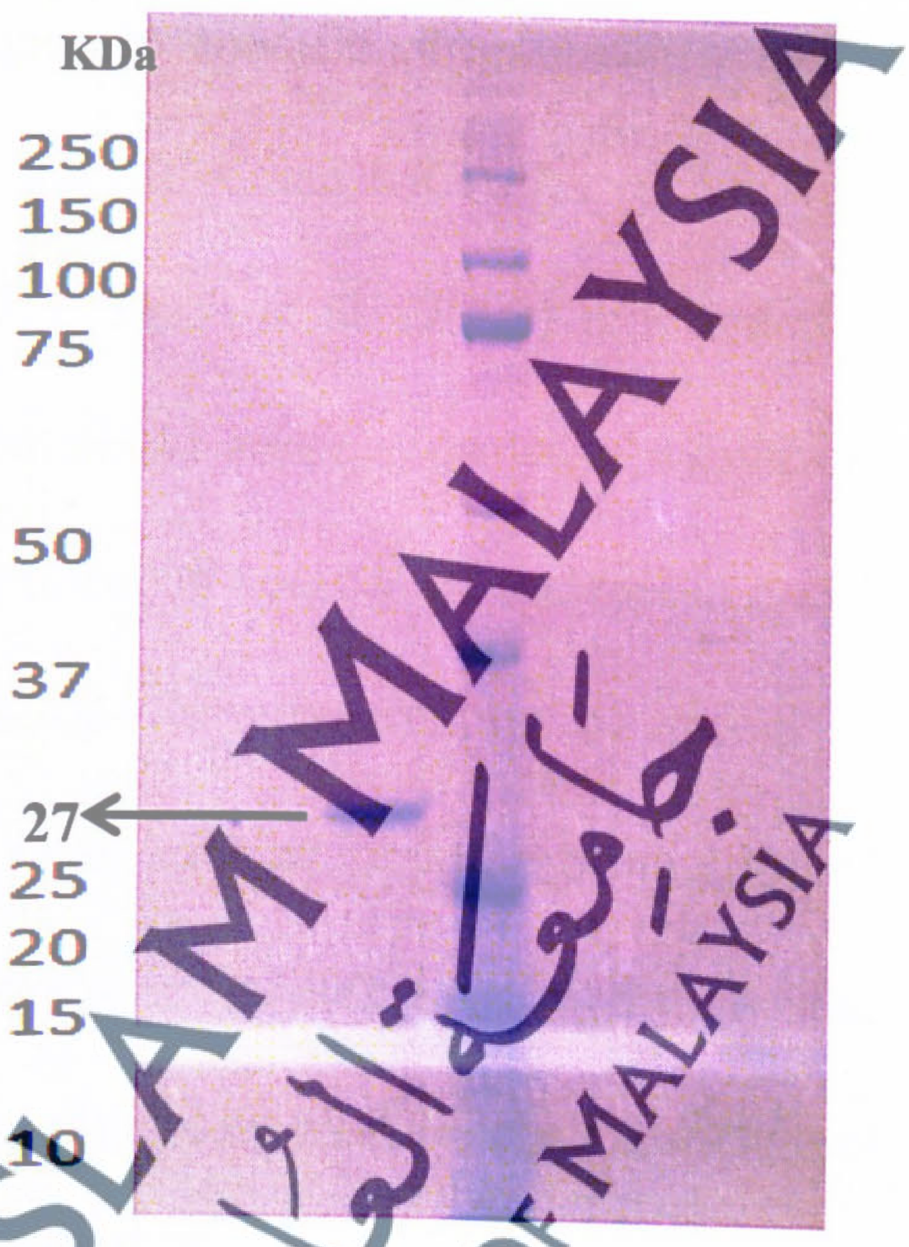


Figure 16: SDS12.5% PAGE of the Purified Enzyme from *L. paracasei* CF1



4.3 DISCUSSION

In this study the types of nitrogen source and concentration affected MCA and PA of two LAB isolates identified as *P. acidilactici* SH isolated from "Belacan" and *L. paracasei* CF1 isolated from Pekasam showed maximum of MCE production after 48 h at 30°C of cultivation. The use of 0.5% casein as nitrogen source in MCE production media recorded MCA of 75 SU/ml and MCA/PA of 37.5 for *P. acidilactici* SH. However, *L. paracasei* CF1 recorded lower MCA of 57 SU/ml and MCA/PA of 21. Production of microbial milk-clotting enzyme (MCE) is affected by different substrates in the enzyme production media (Sato et al., 2004; Merheb-Dini et al., 2010).

The environmental conditions also affect the production of extracellular proteolytic enzymes in the repression or creation of the enzyme by specific compounds (Secades & Guijarro, 1999). Nitrogen sources influenced the MCA and MCA/PA of MCE of microorganisms (Sannabhadti & Srinivasan, 1977). Shieh et al. (2009) reported that the excess of the nitrogen source concentration could repress protease synthesis. Foucaud et al. (2008) reported that changing the nitrogen sources of the media could be used to control the proteolytic activity of LAB. Supplementation of casein or peptides in the enzyme production media can act as an inducer for MCE production of some bacteria (Porto et al., 1996). Llorente-Bousquets et al. (2008) reported that three different extracellular proteolytic enzymes were produced by *Pediococcus acidilactici* ATCC 8042 based on the substrates in enzyme production media.

Concentration of nitrogen source is very an important extracellular proteinase. Joo et al. (2002) observed optimally produced by *Bacillus horikoshii* when 1% casein was added in the medium. Yegin et al. (2010) reported a maximum MCA of 130 SU/ml when 0.5% of casein concentration was used as a nitrogen source in production media. Handel and Fraile (1984) also observed that *M. mucedo* produces maximum MCA when the casein concentration is between 0.5 to 0.7% (w/v). However, in this study MCA was not observed in the supernatant of both isolates of *P. acidilactici* SH and *L. paracasei* CF1 when grown in MRS broth media without additional nitrogen supplementation. In earlier reports, it was found that different nitrogen sources such as soybean meal, casamino acid, and peptone were effective medium ingredients for the protease production by *Bacillus* species (Mehrotra et al. 1999; Joo et al., 2002; Puri et al., 2002; Joo & Chang, 2005; Patel et al., 2005; Chu, 2007).

The MCA obtained in this study for both isolates of *P. acidilactici* SH and *L. paracasei* CF1 were higher than MCA obtained from the isolate of *E. faecalis* TUA2495L (21 SU/ml) in previous study by Sato et al. (2004). However, Dutt et al. (2008) reported a maximum MCA of 120 SU/ml from *B. subitls* when 1.5% casein concentration was used as nitrogen source and decreased to 81 SU/ml when the concentration of casein increased to 2%.

In addition casein played an important role in microbial rennet production under both solid state fermentation and submerged fermentation conditions in the case of *R. miehei* maximum MCA was obtained when 1.5% (w/v) casein was used as nitrogen source in the production media (Silveira et al., 2005). Khademi et al. (2013) and De Lima et al. (2008) reported that rennet obtained from *M miehei* NRRL 3420 produced the highest MCA when 0.4% casein concentration was used and reduction in MCA when casein concentration was increased to 0.8% in enzyme production media. Therefore, each nitrogen source may contain the required amino acid either in insufficient amounts to induce activity in optimum concentrations for initiation of enzymatic synthesis. Using inorganic nitrogen source as the sole nitrogen source caused a significant reduction in the protease yield. This might due to the release of ammonia from these inorganic nitrogen sources (Uyar et al., 2011). The MCA and PA of *M miehei* NRRL 3420 has been reported to be affected by nitrogen sources (Moon and Parulekar, 1991; Chu, Lee and Li, 1992; Sato et al., 2004; Patel et al., 2005). The ratio of MCA/PA can be used as an index to substantiate the sufficiency of MCE to be used as calf rennet substitute (Merheb et al, 2010). Production of MCE is affected by different substrates (Merheb et al., 2010). Therefore, addition of casein 0.5%

concentration for *P. acidilactici* SH and 1% for *L. paracasei* CF1 in the enzyme production media contained sufficient amount of the amino acids required for the synthesis of MCE

The purification of the enzymes by ammonium sulphate remains a common use, for the concentration of the enzymes from microbes (Lee et al., 2002; Cheng et al., 2010) because of its high solubility, cheaper cost and stabilizing effect on most enzymes as well as can be used in acidic and neutral pH solutions (Rifaat et al., 2005). Ammonium sulphate precipitation, Sephadex G 50 filtration and C M sephadex chromatography are employed for the purification of protease. The crude MCE from *P. acidilactici* SH and *L. paracasei* CF1 was purified by ammonium sulfate (60% saturation) and gel filtration by using Sephadex G-50 fine the purified MCE of *P. acidilactici* SH and *L. paracasie* CF1 both showed single band of molecular weight 30 KDa and 27 KDa by SDS-PAGE. Wu et al. (2013) reported that two major bands between 25 KDa and 30 KDa were present in MCE from *B. natto*. Also El-Sayed et al. (2013) obtained a band of molecular weight of 30 KDa from *Brassica napus* Seeds. However, the result from Sato et al. (2004) was slightly different; they reported a band of molecular weight of 34KDa produced by *E. faecalis* TUA2495L. Furthermore, Xing et al. (2012) reported that *L. casei*-D1-1 produced a band with molecular weight of 35 KDa.

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Previous researchers focused more on isolating bacteria from environments that could express high MCE and high ratio of MCA to PA. However, this is the first study on the milk-clotting enzyme produced by LAB from Malaysian fermented food.

4.6 CONCLUSION

Several attempts have been made to substitute calf rennet because of limited supply and increasing high prices. Hence, the interest in using enzymes from microbial sources from the results obtained in this study, casein is considered the most effective nitrogen source in production media of MCE compared to tryptone soya, tryptophan and trypticase peptone. The concentration of nitrogen source and type of nitrogen sources in the enzyme production media of both bacteria showed a high significant difference ($P<0.05$) between MCA and concentration of nitrogen sources and between MCA and types of nitrogen source also observed that partial purification by ammonium sulphate and gel filtration increasing the specific activity of enzyme. Therefore MCE from *P. acidilactici* SH can be used to substitute the rennet in dairy industry.