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RESEARCH PAPER

Purification and characterization of an extracellular protease produced by *Pediococcus pentosaceus* isolated from fermented foods

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Abstract
An extracellular protease, producing lactic acid bacteria (isolate J3) was obtained from fermented food. It was identified as *Pediococcus pentosaceus* based on biochemical properties and 16S rRNA gene sequencing. Cultivation conditions for optimized protease production by this strain were investigated. Maximum protease production was achieved after 20 h of incubation at 30°C with initial pH value 7 of the medium. The protease was purified to homogeneity by ammonium sulphate precipitation, ion-exchange chromatography and gel-filtration with 28.08-fold increase in specific activity, and the molecular weight was estimated as 20 kDa by SDS-PAGE electrophoresis. The protease showed the highest activity at pH and temperature of 4 and 35°C, respectively. The enzyme activity was enhanced with Ca^{2+}, but inhibited by Fe^{3+} and Zn^{2+}. The purified protease was inhibited by EDTA and SDS indicating that it was an acidic metalloprotease. These properties make this protease an ideal choice for application in food industries.

1. Introduction

In the Peninsular Malaysia and Northern Borneo Island of Malaysia, ethnic fermented fruit and vegetable products (e.g., Bambangan, Jeruk, Tempoyak and Gai choy) are prepared from highly perishable fruits and vegetables for consumption, and many lactic acid bacteria (LAB) such as *Lactobacillus*, *Enterococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus* and *Streptococcus*, are found in them (Ajibola *et al*., 2023). LAB are fastidious in their amino acid requirements and there is apparent evidence that LAB are well equipped with proteolytic activities to procure peptides and amino acids to meet the nutrient requirements for their growth (Kieliszek *et al*., 2021). In addition, proteolytic activities of LAB have a strong effect on the sensory properties of fermented food products such as the formation of the characteristic flavour and taste (Sun *et al*., 2019).

In the production of LAB proteases, several factors such as growth medium (i.e., carbon source, nitrogen source, and mineral salts) and culture conditions (e.g., incubation time, pH, temperature, initial inoculum, and oxygen requirement) should be considered, and these factors are varying from strain to strain (Yadav *et al*., 2024). Hence, optimizing the growth of bacterial strains and culture conditions can significantly increase the production of the enzyme worldwide.

In general, purification is one of the essential steps for the study of proteolytic enzymes, mainly to separate the targeted enzymes from other proteins and non-protein parts of the solution, leaving them free of possible contaminants (e.g., bacterial spores, remnants of culture media or nucleic acids), maintaining their chemical integrity and biological activity (Wang *et al*., 2022; Yadav *et al*., 2024). Even though the role of LAB in the production of fermented foods and the alkaline proteases production is well documented (Mustafa *et al*., 2020; Bansal *et al*., 2021), but limited information is available concerning acidic proteases and their productions, especially from *Pediococcus pentoseceus*. Therefore, the objective of this study was to isolate and identify *P. pentoseceus* from fermented foods. Also, to purify and biochemically characterize the extracellular protease from *P. pentoseceus*, which included molecular mass, pH and temperature activity and stability, as well as metal ions and inhibitors.

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Table 1. Proteolytic activity of LAB isolated from fermented foods.

Food samples	Isolate no.	LAB isolate	Proteolytic activity
Bambangan	1	Bm1	+
	2	Bm2	+
	3	Bm3	-
Buah kepayang	4	Bk1	-
	5	Bk2	+
	6	Bk3	+
Dabai	7	Db1	+
	8	Db2	-
	9	Db3	-
Gai choy	10	Gy1	+
	11	Gy2	+
	12	Gy3	-
Jeruk	13	J1	+
	14	J2	+
	15	J3	+
Kedondong	16	Kd1	+
	17	Kd2	-
	18	Kd3	-
Mandai	19	Man1	+
	20	Man2	+
	21	Man3	-
Tempoyak	22	Tp1	+
	23	Tp2	-
	24	Tp3	+

Note: "+", proteolytic activity was detected on SMA plate; "-", no proteolytic activity was detected.

2. Materials and methods

2.1 Sample collection and isolation of LAB

Different types of fermented vegetables and fruits, as listed in Table 1, were collected from Sabah and Sarawak. Samples of 10 g were homogenized with 90 mL sterile peptone water. After a serial dilution to 10^{-8}, bacterial solution of 0.1 mL was spread on de Mann Ragosa Sharpe (MRS; Merck, Darmstadt, Germany) agar plate and incubated at 30°C for 48 h. The isolated pure individual colonies were stored in 20% (v/v) glycerol at -80°C for further studies.

2.2 Measurement of protease activity and protein content

The extracellular protease screening of LAB isolates was performed using skim milk agar (SMA; Merck, Darmstadt, Germany) as previously described (Masi *et al*., 2021). Isolates seen with a clear zone were proceeded for protease activity assay (Pillai *et al*., 2024) using 0.1 M acetate buffer (pH 5.0) as enzyme solution and azocasein as substrate. Protein content was determined by Bradford assay using bovine serum albumin (BSA) as standard (Kielkopf *et al*., 2020), and absorbance (OD) at 280 nm was used to monitor protein concentration during column chromatography.

2.3 Characterization of isolated LAB

The selected isolate was subjected to identification (Table 2) as previously described (Goa *et al*., 2022). The API 50 CHL kit (BioMérieuxÒ SA, Marcy-l'Etoile, France) was used for phenotypic identification, and the results were analysed with the ApiwebÒ identification software.

For molecular identification, QIAamp DNA mini kit (Qiagen, Hilden Germany) was used for genomic DNA extraction, meanwhile, 16S universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGCTACCTTGTTAGCGACTT-3') were used for 16S rRNA gene PCR amplification and sequencing. Analysis of the 16S rRNA gene sequences was performed by BLAST searches of the NCBI database.

2.4 Optimization of protease production

The production of protease by cultivation were as follows: 2.0% (v/v) of 20 h bacterial culture was inoculated into 10 mL MRS broth and incubated at 30°C for 24 h. The cultivation conditions in MRS medium were sequentially optimized as follows: incubation temperature (10, 15, 30, 37, 45 and 50°C) and initial pH of MRS medium (2, 3, 4, 5, 6, 7, 8 and 9). Time course of cell growth and protease production was performed using 1 L MRS medium (pH 7.0), and 5 mL of the culture was withdrawn at 2 h intervals, up to 36 h of incubation for the determination of bacterial cell count, cell density (OD 600 nm), pH of the culture medium and protease activity. All experiments were carried out three times independently with technical triplicates.

2.5 Purification of protease

For crude extracellular protease preparation, the culture was centrifuged at 10,000x g for 15 min at 4°C after cultivated in optimum conditions. Ammonium sulphate precipitation using 70% saturation was carried out, and the precipitate was centrifuged at 8,000x g for 15 min at 4°C, re-dissolved in 50 mM sodium acetate buffer (pH 5.0) [known as buffer A]. The enzyme solution was loaded onto a HiPrepä 26/10 desalting column, equilibrated with buffer A at a flow rate of 7.5 mL/min. The desalted enzyme solution was subjected to a DEAE-Sepharoseä FF column (which equilibrated with buffer A) and the bound proteins were eluted with a linear gradient of 1 M NaCl in buffer A at a flow rate of 1 mL/min. The active fractions were pooled and applied to a Sephadexä G-75 column, equilibrated and eluted with 10 mM sodium acetate buffer (pH 5.0) containing 0.1 M NaCl at a flow rate of 0.4 mL/min. The active fractions were pooled and concentrated for further studies.

2.6 Characterization of purified protease

Molecular weight was analyzed with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% resolving gel and 5% stacking gel (Laemmli, 1970).

Effect of pH and temperature on protease activity and stability were determined as previously described (Wang *et al*., 2022) with slight modification, where the optimum pH of protease activity was estimated within pH 3.0-6.5, and buffer systems at pH 3-10 [sodium citrate buffer (pH 3-5), phosphate buffer (pH 6-8) and glycine-NaOH buffer (pH 9-10)] were used for pH stability determination. Meanwhile, the optimum temperature of protease activity was evaluated at various temperatures (20, 25, 30, 35, 40 and 45°C), and temperatures ranging from 20-80°C were used for thermal stability determination.

Different metal ion with concentration of 1 mM and 10 mM, and inhibitors with concentration of 5 mM, were used to investigate the effects on protease activity (Table 4). The highest activity of protease was considered 100%, and all assays were performed in triplicates.

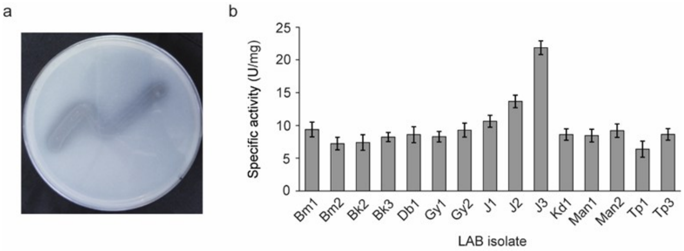

Figure 1. Screening of protease producing LAB isolates. (a) Claer zone on SMA plate. (b) Protease activity of LAB isolates. Results are means from triplicate assays, and error bars show the standard deviation (SD).

3. Results and discussion

A total of 24 LAB isolates were isolated from the fermented foods (Table 1), and were preliminary screened for extracellular protease on SMA plate. The presence of protease from LAB was indicated by a clear zone forming around the colonies on SMA plate (Figure 1a), and all positive isolates were selected for enzyme quantification using azocasein. Among the 15 isolates tested, J3 isolate from 'jeruk' sample exhibited the highest proteolytic activity (Figure 1b), indicating as a versatile extracellular protease producer. Depending on the species, subspecies or strain, LAB show very diverse proteolytic activity which related to the utilization of energy sources, providing the cells with essential amino acids during their growth.

Table 2. Biochemical characteristics of J3 isolate.

Characteristics tested	Reaction results
Gram-staining	+
Catalase test	-
Arginine hydrolysis	+
pH tolerance:	
pH 4.2	+
pH 4.8	+
pH 7.5	+
pH 8.5	+
pH 9.6	-
Temperature tolerance:	
10°C	-
15°C	-
30°C	+
37°C	+
45°C	+
60°C	-
NaCl tolerance:	
4.0% NaCl (w/v)	+
5.0% NaCl (w/v)	+
6.0% NaCl (w/v)	+
6.5% NaCl (w/v)	+

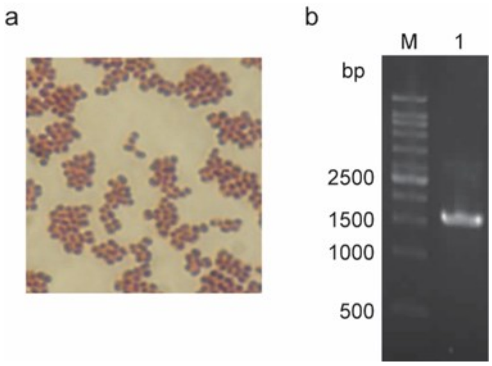

Figure 2. Cell morphology and 16S rRNA of J3 isolate. (a) Gram-staining micrograph, 100x magnification. (b) Agarose gel electrophoresis of 16S rRNA gene. Lane M: 1kb DNA ladder; Lane 1: 1500 bp PCR product.

The J3 isolate was selected for morphological, physiological, and biochemical test identification. As shown in Table 2, isolate J3 was a Gram-positive, catalase-negative, hydrolyzed arginine, non-motile, and non-spore forming bacteria. The isolate was capable to grow in a wide range of pH (4.2-8.5), temperature (30-45°C), and salt concentration [4.0-6.5% (w/v)]. The colony appeared in the formation of tetrads via cell division in two perpendicular directions in a single plane as demonstrated by microscopy (Figure 2a), and was able to consume 17 of out 50 carbon sources in the API 50 CHL kit (data not shown). For molecular identification, amplified PCR 16S rRNA gene (Figure 2b) was sequenced, and Blastn result showed 98.21% homologous to the nucleotide of *P. Pentosaceus* strain SA14 (ON103325.1). Hence, isolate J3 was designated as *P. Pentosaceus* J3. Previously, 16S rRNA was demonstrated with high similarity of 99% to identify *Levilactobacillus brevis* and *Lactiplantibacillus plantarum* from fermented goat milk butter (Bentahar *et al*., 2024).

Parameters such as temperature and initial pH of the medium that affects the microbial cell growth and protease production were investigated. Enzyme production was detected at temperatures ranging from 30 -45°C and achieved maximum activity at 30°C; no bacterial growth and protease activity were observed at ≤15°C and ≥50°C (Figure 3a). Protease production of *P. pentosaceus* J3 was found active within the initial pH range of 4.0-8.0, with optimum cell growth and enzyme activity at pH 7.0 (Figure 3b). For the time course study, lag phase of *P. pentosaceus* J3 growth was short (approximately 2 h), where exponential phase began drastically after 4 h of cultivation, and reached stationary phase after 16 h of incubation (Figure 3c). The pH of the

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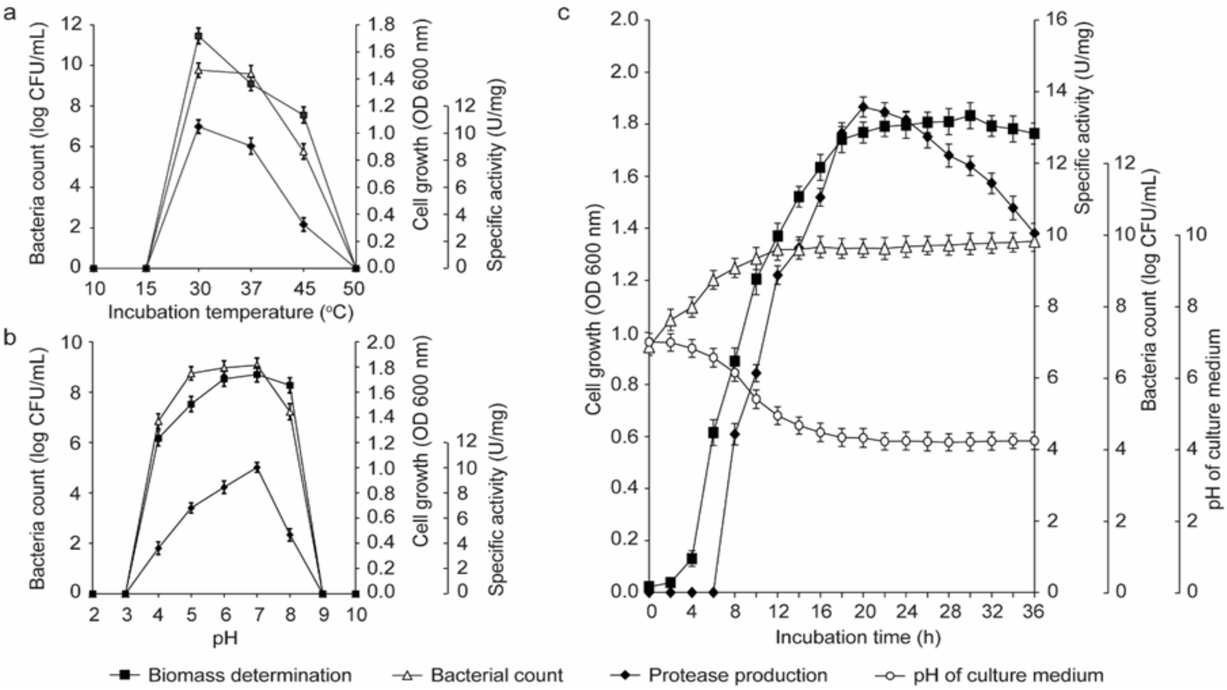

Figure 3. Optimization of protease production from *P. pentosaceus* J3 isolate. (a) Effect of incubation temperature. (b) Effect of initial pH. (c) Time course of cell growth.

growth medium decreased toward acidic pH and remained stable at pH 4.4 after 18 h of incubation, which could be due to the production of organic acid from glucose fermentation. The proteolytic activity was undetectable during the early-exponential growth phase but increased rapidly from the mid of the exponential phase (after 6 h), and the maximum protease production was detected at 20 h of incubation.

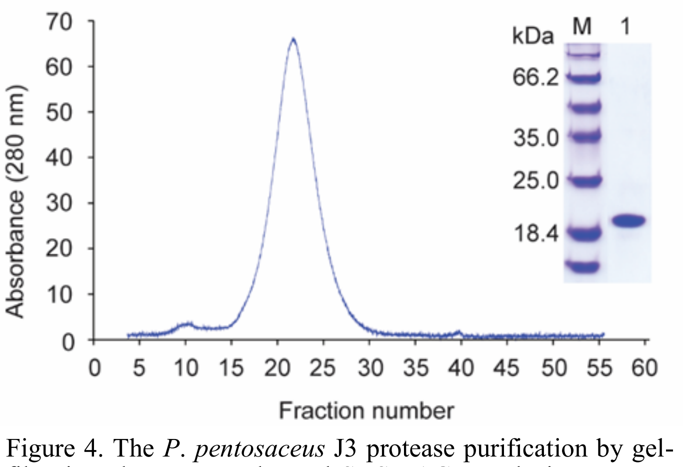

Figure 4. The *P. pentosaceus* J3 protease purification by gel-filtration chromatography and SDS-PAGE analysis. Lane M: protein molecular weight marker; Lane 1: sample after gel-filtration chromatography purification.

The purification of *P. pentosaceus* J3 extracellular protease was performed by ammonium sulphate precipitation, HiPrep desalting, DEAE-Sepharose ion-exchange chromatography and Sephadex G-75 gel-filtration chromatography. The results of 70% ammonium sulphate saturation showed that 20.4 mg protein with 62.6% yield were obtained (Table 3). After three-steps purification using fast protein liquid chromatography (FPLC), the purified protease appeared as a single peak on Sephadex G-75 (resulting in 28.8% yield and 28.08-fold purification), and as a single band on SDS-PAGE (Figure 4). The molecular weight of the enzyme was estimated to be 20 kDa. Similarly, an extracellular protease from *P. pentosaceus* with molecular weight of 29.6 kDa was purified by DEAE-Sepharose and Sephadex G-75 to 25.6-fold purification (Sun *et al.*, 2019).

The purified protease was found to be active between pH 3.0 and 5.0, with the highest activity at pH 4.0, and almost no activity was detected at pH 6.5 (Figure 5a). Based on this observation, *P. pentosaceus* J3 extracellular protease could be classified as an acidic protease. The enzyme was highly stable over acidic pH range (pH 4.0-6.0) and maintaining over 90% of its original activity, while less than 40% of its original activity was retained beyond pH 9.0 (Figure 5b). The effect of temperature on the proteolytic activity showed that more than 80% of the relative activity was detected between 30°C and 40°C, and the highest activity (100%) was obtained at 35°C (Figure 5c). Besides, the purified

Table 3. Purification of protease from *P. pentosaceus* J3 isolate.

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude supernatant	2.03×10^5	78.2	2.6×10^3	100	1
$(NH_4)_2SO_4$ precipitation	1.27×10^5	20.4	6.2×10^3	62.6	2.38
HiPrep desalting	1.02×10^5	13.8	7.4×10^3	50.2	2.85
DEAE-Sepharose	9.15×10^4	5.5	1.7×10^4	45.1	6.54
Sephadex G-75	5.84×10^4	0.8	7.3×10^4	28.8	28.08

Note: Yield (%) = (Total activity/Total activity of culture medium supernatant) × 100; Purification fold = Specific activity/Specific activity of crude extract.

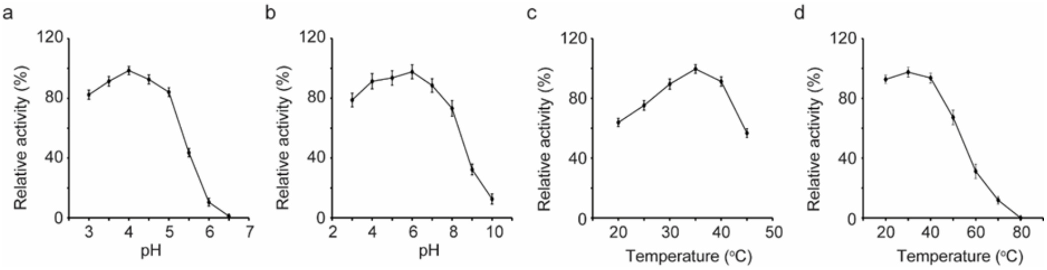

Figure 5. Effect of pH and temperature on the protease activity (a and c) and stability (b and d).

Table 4. Effect of metal ions and inhibitors on the protease activity.

Reagents	Relative activity (%)	
Metal ion:	Concentration (1 mM)	Concentration (10 mM)
$CaCl_2$ (Ca^{2+})	106.5 ± 1.8	124.1 ± 1.2
$CoCl_2$ (Co^{2+})	101.2 ± 0.9	103.7 ± 0.7
$FeCl_3$ (Fe^{3+})	44.3 ± 1.1	21.4 ± 0.8
KCl (K^{+})	103.8 ± 1.7	102.6 ± 2.1
$MgCl_2$ (Mg^{2+})	91.7 ± 0.8	82.5 ± 1.1
$MnCl_2$ (Mn^{2+})	95.1 ± 1.3	90.2 ± 0.9
$ZnCl_2$ (Zn^{2+})	67.2 ± 0.6	26.5 ± 0.5
Inhibitors:	Concentration (5 mM)	
dithiothreitol (DTT)	93.1 ± 0.9	
ethylenediaminetetraacetic acid (EDTA)	50.4 ± 1.1	
iodoacetic acid (IAA)	88.8 ± 1.7	
phenylmethylsulfonyl fluoride (PMSF)	98.2 ± 0.8	
SDS	62.6 ± 0.6	

enzyme appeared to be stable at temperature range of 20-40°C, but the activity decreased dramatically when the temperature increased above 40°C (Figure 5d). Next, the effect of metals ion and protease inhibitors on purified protease were evaluated (Table 4). The enzyme was strongly inhibited by 1 and 10 mM of Fe^{3+} and Zn^{2+}. Obviously, cation Ca^{2+} was found to stimulate the activity at high concentration of 10 mM compared to low concentration of 1 mM. In fact, calcium ions are well known stabilizers which protect many enzymes from conformational changes (Zhang *et al*., 2019). Meanwhile, the nature of the acidic protease was determined by different inhibitors such as EDTA (metalloprotease inhibitor), IAA (cysteine protease inhibitor) and PMSF (serine protease inhibitor), where EDTA decreased the enzyme activity to 50%, suggesting that *P. pentosaceus* J3 extracellular protease belongs to metalloproteases group. Overall, *P. pentosaceus* J3 extracellular protease may have potential for application in food processing to improve food quality.

Conclusion

Production and characterization of an extracellular protease produced by isolated *P. pentosaceus* J3 was studied. A 20 kDa extracellular protease was purified using ammonium sulphate precipitation, ion exchange and gel filtration chromatography. It was revealed that the enzyme was a metal chelator sensitive acidic protease with effective stability in pH 4.0-6.0. In this context, the biochemical properties of the protease may contribute to the protein degradation, as well as the formation of flavour and the development of textural in fermented food, suggesting that *P. pentosaceus* J3 can be used as a potential starter culture in food fermentation.

Conflict of interest

The authors declare no conflict of interest.

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