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Cost-Effective Strategy and Feasibility for Amylase Production from Okara by *Bacillus subtilis* J12

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Abstract: Low-cost enzyme production is considered a feasibility factor in enzyme commercialization. Okara, a high-nutritional agro-industrial residue from soybean processing, was performed as a medium for bacterial amylase production to save costs and increase productivity. This study aimed to produce, characterize, activate amylase, and evaluate the material cost for media from okara. Under solid-state fermentation (SSF) of okara without pretreatment, *Bacillus subtilis* J12 could produce 983 U/g of amylase within 24 h. *Bacillus subtilis* J12 amylase had optimal activity at pH 6.0 and 50 °C and was stable at a moderate temperature for up to 120 min. Identified as a metalloenzyme, the activity was improved by ferric ions. The purification of amylase resulted in two fractions which contained at least two types of amylases. Compared with other producers, the production was evaluated using low-cost media without additional supplementations. Based on the productivity, characteristics, and evaluation, *Bacillus subtilis* J12 amylase was potentially commercialized, had economic value, possessed energy-saving features, and could be applied for industrial use.

Keywords: amylase; *Bacillus subtilis*; okara; activation; cost

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1. Introduction

The development of low-cost processes is recognized as a crucial aspect and challenge in enzyme commercialization. To reduce production costs related to fermentation media, many studies have focused on utilizing agro-industrial residues as alternative media. Agricultural wastes such as rice husk, wheat straw, pearl millet straw, moong husk, sugar cane bagasse, oat straw, maize straw, potato, orange, and pomegranate peels have been reported as media supplements in alpha-amylase production [1]. On the other hand, the cost of media composition was rarely evaluated, even though many agro-industrial residues were studied as media for amylase production. In fact, it is important for industrial consideration based on the feasibility and disadvantages.

Okara, a by-product of soy milk and tofu production that is rich in nutritional composition, has the potential to serve as an alternative bacterial cultivation medium to produce enzymes. Okara could serve as a carbon, nitrogen, and other nutrition source to support bacterial growth. Moreover, other studies have reported that okara remains high in dietary fiber, protein, unsaturated fatty acids, isoflavones, minerals, and oligosaccharides [2]. Okara comprises 50% dietary fiber, 25% protein, 10% lipid, 3.8–5.3% carbohydrates, and 3–4.5% ash in 100 g of dry matter [3]. Several carbohydrates, such as carbon sources, play a role as stimulants from 1.0–6.0% to optimize amylase production [1]. On the other hand, the high global annual production of okara led to the high costs of okara disposal. For example, Japan has a huge okara production and spent approximately USD 145 million on the disposal of 800,000 metric tons of okara [4]. In other cases, okara is possibly unprocessed and improperly disposed in the environment, leading to new environmental

issues. Consequently, okara utilization is promising to produce eco-friendly products such as enzymes to reduce waste and save our environment.

Fungi and bacteria are the primary producers of amylase and are extensively used in various industries. Amylase is commonly used to convert starch or other polysaccharides into small units of sugars, such as glucose and maltose. On an industrial scale, amylase is utilized as a biocatalyst for various purposes, including starch processing, liquefaction, and maltose syrup production, as well as in baking, biofuel production, feed, paper, textile, detergent, and pharmaceutical industries [5–9]. *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus cereus*, and *Anoxybacillus amylolyticus* are among the most common amylase-generating bacteria used in industry [10]. In addition, a previous study reported that *Bacillus subtilis* J12 can produce thermophilic CMCase and xylanase through submerged fermentation and are recognized as Gram-positive bacteria, have a rod shape, are motile, and the colony is circular, raised, translucent, and smooth [11]. The *B. subtilis* has a single-cell membrane that facilitates protein secretion, simplifies downstream processing, and reduces process costs. These strain characteristics fulfill the generally recognized as safe (GRAS) requirement [12]. This suggests the potential for exploring other enzymes that act on polysaccharides from *B. subtilis* J12, such as amylase.

In enzyme production, the fermentation method plays an important role, which influences enzyme activity and productivity. Thus, solid-state fermentation (SSF) is the preferred method for amylase production due to okara being is stored in a dried condition to extend its shelf life and prevent contamination. Under solid-state fermentation, okara support microbial growth on the surface and, inside, a solid matrix in the absence of free water [13]. This method yields a higher enzyme concentration, increases enzyme efficiency, and exhibits different enzyme characteristics compared to submerged fermentation [14,15]. Solid-state fermentation involves positioning the cultured microorganisms close to the substrate. It facilitates microbial physiological and physicochemical factors that influence the fermentation process [16].

Nevertheless, some evidence showed that amylase produced by bacteria exhibited lower enzymatic activity and distinct characteristics compared to fungal amylase. This may be attributed to the unique enzyme conformation of bacterial amylase, which could affect its catalytic activity. Enhancing enzyme function and activation through adjustments in its conformation is achievable, due to its innate properties and characteristics. Therefore, this study aimed to produce, characterize, and activate amylase from the solid-state fermentation of okara by *B. subtilis* J12, as well as evaluate the material cost for media production.

2. Materials and Methods

2.1. Bacterial Strain and Inoculum Preparation

Bacillus subtilis J12, isolated from hot spring water (GenBank accession number KP996492), was kept in the culture collection of the Faculty of Technology, Khon Kaen University, Khon Kaen, Thailand. The culture was refreshed in nutrient broth for 18–24 h at 37 °C before separating the cell by centrifugation at 10,000 rpm for 2 min (AS One, Osaka, Japan). The cell pellet was washed and diluted in sodium chloride (0.85% v/v). Furthermore, optical density was adjusted to 1.00 (4.7×10^5 CFU/mL) at 600 nm using a UV-VIS spectrophotometer (AS One, Osaka, Japan).

2.2. Media Preparation

Okara was rinsed with water to clean it and was placed in the hot air oven at 60 °C until dry. Dried okara was kept in a closed pack at room temperature. Before fermentation, the dried okara was weighed and autoclaved at 121 °C for 15 min.

2.3. Production of Amylase

Production of amylase was carried out by solid-state fermentation (SSF). In brief, sterile distilled water was added to the okara and the moisture content was maintained (65–70% v/w). The inoculum of *B. subtilis* J12 (10% of the initial media weight v/w) was

inoculated into okara and it was mixed manually before incubating at 37 °C for 48 h. The fermented okara was collected at 12, 24, 30, 36, and 48 h. Crude amylase was extracted by adding sterile distilled water into the fermented samples in a ratio of 1:7 (w/v) and waiting for 30 min. Crude amylase was filtered twice using Whatman No. 1 filter paper (Cytiva, Little Chalfont, UK), followed by centrifugation (SIGMA, Osterode am Harz, Germany) at 10,000 rpm, 4 °C for 10 min to obtain the supernatant.

2.4. Determination of Amylase Activity

The amylase activity was determined according to Bernfeld [17], with slight modifications. Briefly, 0.25 mL of crude enzyme was added to 0.25 mL of soluble starch (1% w/v) in 0.5 mM sodium phosphate buffer (pH 4.5). The mixture was incubated at 37 °C for 10 min, followed by adding 0.5 mL of 3,5-dinitrosalicylic acid (DNS) solution [18]. A control of the reaction was performed by mixing the substrate and DNS solution before adding the enzyme. The test tube was capped and immediately boiled for 5 min. Furthermore, the mixture was cooled in cold water and 5 mL of distilled water was added. Absorbance was read at 540 nm against the blank solution without the enzyme. D(+)-glucose (KemAus, Cherrybrook, Australia) solution was prepared as the standard. One unit of amylase activity is defined as the amount of enzyme required to release 1 µmol of reducing sugar as glucose per minute, under assay conditions. Amylase activity and productivity were calculated by the formulae as follows.

$$\text{Activity (U/mL)} = ((A \times B) \times 1/C \times 1000) \times 1/D \times 1/E \quad (1)$$

$$\text{Activity from okara (U/g)} = (1) \times F \;/\; G \quad (2)$$

$$\text{Productivity} = (2) \;/\; H \quad (3)$$

where A is the concentration of standard equivalent released (mg/mL), B is the total assay volume (mL), C is the molecular weight of the standard used (g/mol), D is the reaction time (minute), E is the volume of enzyme used (mL), F is the dissolving volume (mL), G is the weight of dry substrate (g), and H is the fermentation time (h).

2.5. Characterization of Amylase

2.5.1. Effect of pH on Amylase Activity

Several buffers were used to determine the optimum pH for amylase activity: 0.1 M acetate buffer (pH 4.0 and 5.0), 0.1 M sodium phosphate buffer (6.0 and 7.0), and 0.1 M Tris-HCl buffer (pH 8.0 and 9.0). In brief, 0.5 mL of the enzyme was mixed with a buffer of up to 1 mL of the final volume. Immediately, the enzyme buffer solution was added into soluble starch 1% (v/v) and incubated at room temperature (25 °C) for 10 min before the DNS was added. The activity was tested following the DNS method [18].

2.5.2. Effect of Temperature on Amylase Activity

The reaction between enzyme and 1% (v/v) soluble starch at the optimal pH (Section 2.5.1) was performed at different temperatures (30–100 °C) for 10 min, to determine the optimum temperature. The activity was tested following the DNS method [18].

2.5.3. Thermostability of Amylase Activity

The enzyme was pre-incubated at different temperatures (30–60 °C) and collected for 0–120 min. The enzyme was added into soluble starch 1% (v/v) with the optimal pH (Section 2.5.1) and incubated at 37 °C for 10 min. The relative activity (%) was compared with the activity at 0 min as 100% (control).

2.5.4. Effect of Organic Solvent on Amylase Activity

The enzyme was pre-incubated in organic solvents (methanol, ethanol, propan-2-ol, and n-butanol) at room temperature for 30 min at 10% and 25% (v/v) of the final

concentration of solvents. The absence of an organic solvent was considered as 100% activity and then compared to the relative activity (%). All assays were performed under standard conditions.

2.5.5. Effect of Metal Ion on Amylase Activity

The enzyme was pre-incubated in metal ion solution (Na^+, Ca^{2+}, Mn^{2+}, Mg^{2+}, K^+, and Fe^{3+}) at room temperature (25 °C) for 30 min at 5 mM and 10 mM of the final concentration of the metal ions. The activity in the absence of metal ions as the control was taken to be 100% and compared to the relative activity (%).

2.5.6. Effect of Ferric Ion on Amylase Activity

Different final concentrations of ferric ion (Fe^{3+}) ranging from 5 mM to 25 mM were used for a pre-incubate enzyme for 30 min. The activity in the absence of ferric ions as the control was taken to be 100% and compared to the relative activity (%).

2.6. Hydrolysis Pattern

Analysis was carried out by following Thin Layer Chromatography (TLC) protocols with slight modifications [19]. Briefly, 3 µL of starch hydrolysate was spotted on TLC silica gel 60 F254 (Merck KGaA, Darmstadt, Germany and dried thrice before the mobile-phase movement step. The mobile-phase reagents were formulated by mixing *n*-butanol, propan-2-ol, ethanol, and deionized water in the ratio of 2:3:3:2, respectively. After the mobile phase, the TLC plate was immediately dipped into the color development reagent containing 2% (*w*/*v*) orcinol in 1% (*v*/*v*) sulfuric acid in ethanol. Furthermore, the TLC plate was heated at 90 °C until the colors of the spots were exhibited. Both D(+)-glucose (KemAus, Cherrybrook, Australia) and D(+)-maltose (KemAus, Cherrybrook, Australia) in the concentration of 1 mg/mL (*w*/*v*) were used as standard markers.

2.7. Purification of Amylase

The crude enzyme in the form of supernatant was filtered with 0.45 µm of cellulose acetate membrane (FilTrex, Encinitas, CA, USA) to separate the cells and other particulates. The crude enzyme was partially purified with one-step purification using the NGC Quest 10 Chromatography System (BioRad, Hercules, CA, USA) equipped with the anion exchange column (Mecro-Prep High Q, BioRad, Hercules, CA, USA). The column was equilibrated and washed with deionized water, followed by 0.1 M Tris buffer (pH 8.0) as the initial buffer and eluted by NaCl 1 M at a flow rate of 5.0 mL/min. The fractions were collected and tested for amylase activity at pH 6.0 and 50 °C, followed by measuring reducing sugar by the 3,5-dinitrosalicylic acid (DNS) method. Fractions with the highest activity were chosen to analyze the purity using Native PAGE, SDS-PAGE, and zymography.

2.8. Native PAGE, SDS-PAGE, and Zymography of Amylase

The molecular weight of amylase was estimated using Native PAGE and SDS-PAGE according to Laemmli [20], with slight modifications, and proved the activity of zymography. Native PAGE and zymography were performed under non-denaturing treatment, to maintain enzyme activity. Standard molecular weight of protein (Bio-helix, New Taipei, Taiwan) was used. In brief, fractions were loaded onto Native PAGE and Tris-HCl SDS-PAGE gel (10% separating and 4.5% stacking gel). The electrophoresis was constantly run (100 V; 400 mA) and stained with Coomassie Brilliant Blue R-250 (AppliChem, Darmstadt, Germany).

In addition, Native zymography was carried out by loading fractions into 10% separating gel containing 0.1% (*w*/*v*) soluble starch. After electrophoresis, the gel was gently agitated in 1% (*v*/*v*) Triton X-100 for 30 min and rinsed at least five times with deionized water. The gel was incubated in Tris buffer (pH 7.0) at 37 °C for 1 h. After incubation, the gel was stained with Lugol's iodine solution and the clear zone was determined as the enzyme activity.

2.9. Material Cost Evaluation

Variable material costs including agricultural wastes, chemicals, and media supplements, were calculated for cost evaluation. The cost of agricultural waste was assumed as the free material, while additional ingredients such as the chemicals and media supplementation prices were considered from the available price on the distributor website. Calculations were formulated for cost evaluation based on the total material needed, as follows.

$$\text{Cost per unit (USD)} = (\text{Material cost (USD)}/100\ \text{g}) \times \text{Material used (g)} \quad (4)$$

$$\text{Total cost (USD)} = \Sigma\ \text{Cost per unit (USD)} \quad (5)$$

2.10. Statistical Analysis

The data results were analyzed by one-way analysis of variance (ANOVA) using IBM SPSS version 28.0 (IBM Corp, Armonk, NY, USA). The significance of all data results was determined at a p-value < 0.05.

3. Results and Discussion

3.1. Production of Amylase Under Solid-state Fermentation

The fermentation time is crucial in the production of enzymes that depend on bacterial growth during fermentation. In this study, amylase activity was performed at different fermentation periods (12, 24, 30, 36, and 48 h). As shown in Figure 1, *B. subtilis* J12 produced extracellular amylase of 983 U/g from okara in 24 h, which was determined as the suitable time for amylase production with a productivity of 40.96 U/g per hour. After 30 h fermentation, there was a notable decrease in activity, possibly due to the nutrient components being limited in a long period of fermentation [21].

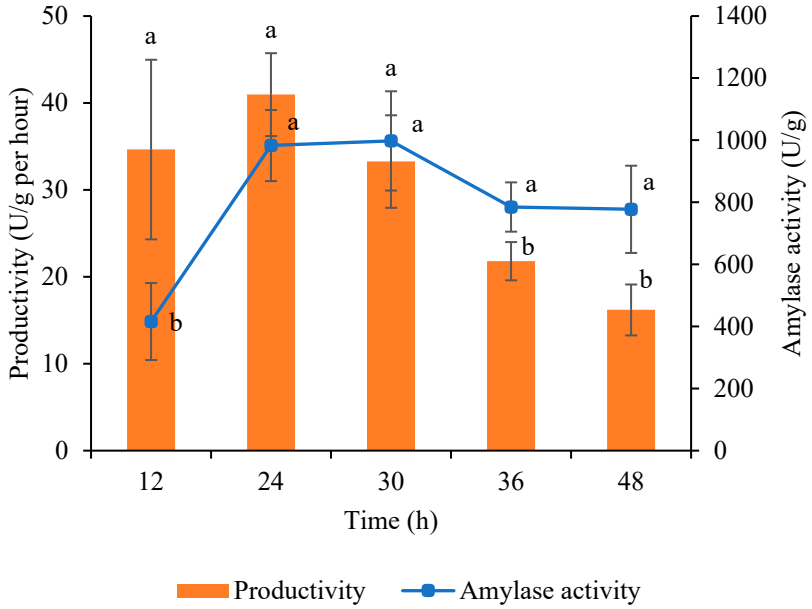

Figure 1. *Bacillus subtilis* J12 amylase activity and productivity from okara in solid-state fermentation. Different superscripts show a significant difference (p < 0.05).

Efficient enzyme production with high enzyme activity and productivity is considered for commercial purposes. The cost of production is a major factor in the total cost of industrial processes. Thus, with high productivity, the cost of production can be minimized [22]. Commonly, the catalytic activity of extracellular amylase produced by bacteria is lower than that of fungal amylase. However, bacteria possess the capability to grow more rapidly

than fungi. Therefore, the fermentation time and media could be optimized to obtain high bacterial enzyme productivity.

As indicated in Table 1, *B. subtilis* J12 exhibited competitive productivity, with faster production. This discovery is an advantage for enzyme manufacturers for fulfilling the high demand for amylase in the industrial sector. With faster production, *B. subtilis* J12 could compete with other amylase producers that utilize agro-industrial residue as a media. Our findings showed that, within 24 h, *B. subtilis* J12 can reach high productivity among bacterial and fungal producers. Hence, this finding and comparison can prove that *B. subtilis* J12 amylase can be potentially developed for commercial purposes.

Table 1. Comparison of amylase production by different agro-industrial residue media.

Producer	Media	Enzyme Activity	Fermentation Time	Productivity (U/h) [2]	Reference
B. subtilis J12	Okara	983 U/g	24 h	40.96	This study
B. amyloliquefaciens	Wheat bran and potato peel	99 U/mL	240 h	0.41	[23]
B. subtilis KR1	Wheat bran	82.6 U/gds	72 h	1.15	[16]
B. velezensis KB 2216	Moong husk and soybean cake	75.78 U/mL	72 h	1.05	[1]
B. tequilensis TB5	Rice bran	37.7 U/mL	72 h	0.52	[10]
Penicillium notatum NCIM 923	Wheat bran	2819.24 U/g	94 h	29.99	[24]
Aspergillus oryzae NRRL695	Soybean husk and flour mill waste	47,000 U/gds	360 h	130.5	[25]
Aspergillus fumigatus SKF-2	Agricultural residues [1]	1523.3 U/gds	300 h	5.08	[26]
Aspergillus oryzae	Oil cake	10,994.7 U/gds	108 h	101.8	[27]

[1] Mixture of sugarcane bagasse, orange peel, and wheat bran. [2] Productivity = enzyme activity/fermentation time.

3.2. Characterization of Amylase

3.2.1. Effect of pH on Amylase Activity

The amylase activity was measured atdifferent pH buffers from 4.0 to 9.0. As shown in Figure 2a, amylase activity was not significantly different at the pH range of 4.0 to 7.0. The optimum pH for amylase activity was 6.0 (sodium phosphate buffer). The enzyme was active more than 80% of the maximum activity in the range of acidic and neutral conditions. However, the amylase activity of *B. subtilis* J12 decreased in the alkali environment and exhibited 49% of the maximum activity at pH 9.0. This corresponded to another study of a novel acidic thermostable α-amylase, which exhibited 72% activity at pH 4.0 and 46% activity at pH 9.0 [8].

3.2.2. Effect of Temperature on Amylase Activity

Amylase activity was tested under different temperatures, from 30 °C to 100 °C. As shown in Figure 2b, the amylase works well in the range of 40 to 70 °C, exhibiting more than 75% of the maximum activity. This finding supported energy saving due to enzyme activation at moderate temperatures. However, the amylase activity declined at temperatures of more than 60 °C. The high temperature might influence the active site of the enzyme; then, the enzyme became inactive or potentially denaturized. A previous study revealed that α-amylase from *Bacillus methylotrophicus* DCS1 reached the maximum activity at 60 to 65 °C [28]. In contrast, α-amylase from *Aspergillus fumigatus* SKF-2 produced under solid-state fermentation exhibited the maximum activity at 30 °C [26].

3.2.3. Thermostability of Amylase Activity

The stability of amylase activity was measured after pre-incubation within 120 min at different temperatures between 30 and 60 °C. Based on the result in Figure 2c, the enzyme was stable at 30 to 50 °C for 120 min and retained more than 80% activity at 50 °C from

the initial pre-incubation time. In addition, the activity gradually decreased during pre-incubation at 60 °C. Thus, it was determined that the optimum amylase activity was at 50 °C. These findings implied that long exposure to high temperature affected *B. subtilis* J12 amylase activity. The enzymes gradually lose their activity at high temperature because proteins in their structure become inactive due to heat or, potentially, through peptide chain hydrolysis, amino acid breakdown, or protein aggregation or denaturation. Other studies reported that α-amylase from *Bacillus velezensis* KB 2216 under submerged fermentation using a combination of agricultural wastes showed a reduction of 50% activity when the temperature increased more than 60 °C [1]. In addition, α-amylase from *Trichoderma pseudokoningii* under solid-state fermentation using orange peels was highly stable at 80 °C for 1 h enzyme incubation [29].

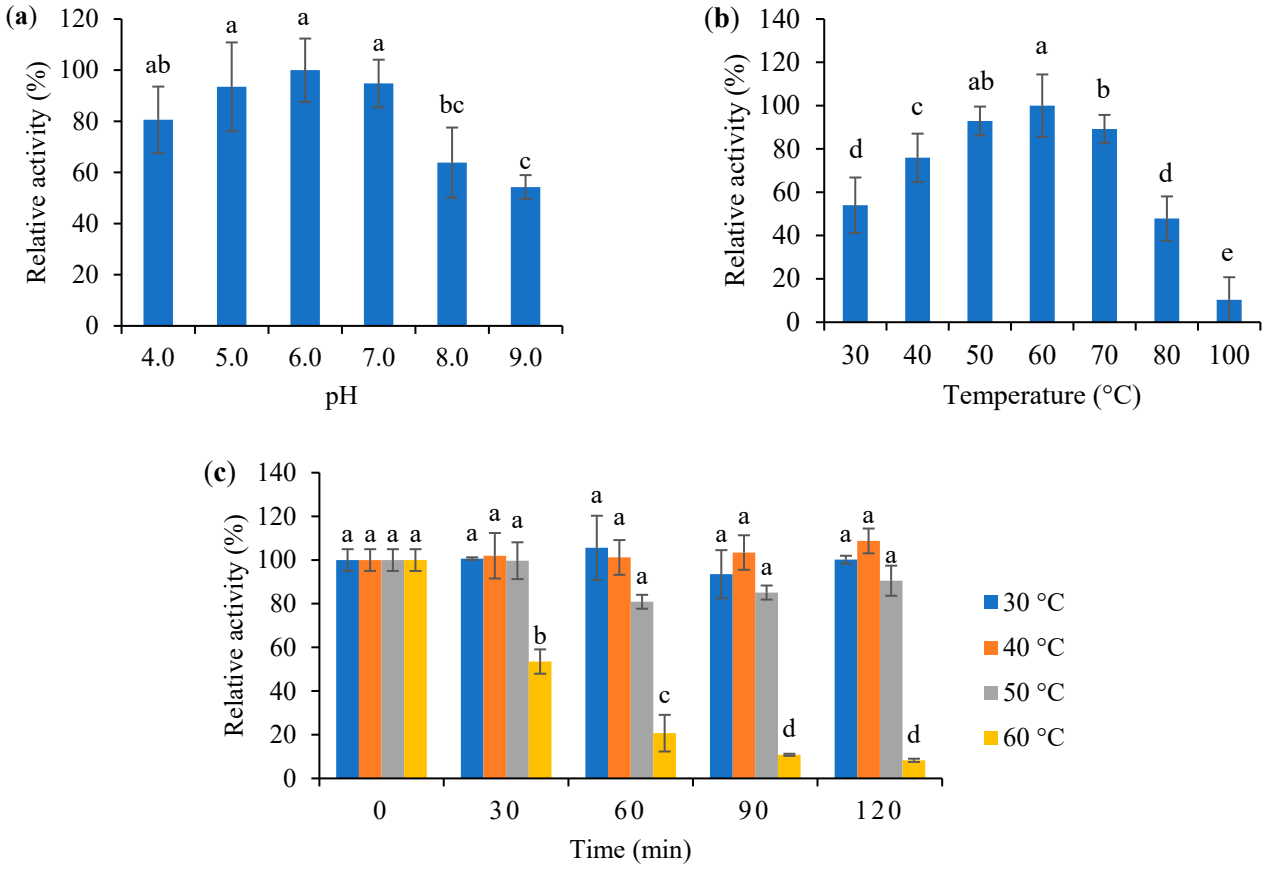

Figure 2. Effect of (**a**) pH and (**b**) temperature on amylase activity and (**c**) thermostability of amylase activity. Different superscripts show a significant difference ($p < 0.05$).

3.2.4. Effect of Organic Solvent on Amylase Activity

The amylase activity was observed after pre-incubating the enzyme in different concentrations of organic solvents. As shown in Figure 3, all organic solvents at concentrations of 10% and 25% significantly decreased amylase activity of *B. subtilis* J12. The enzyme maintained over 80% of its relative activity in the presence of solvents. The polarity change in the enzyme caused by organic solvent disrupts the active-site conformation, making it less effective at catalyzing reactions. Organic solvent influenced the reduction of enzyme catalytic activity due to the conformational change, loss of conformational flexibility and crucial water, thermodynamic stabilization, and interfacial inactivation [30]. Previous studies indicated that higher amounts of methanol, propanol, and butanol led to potent inhibition of amylase activity, which retained 36%, 31%, and loss activity at 30% of organic solvents [31].

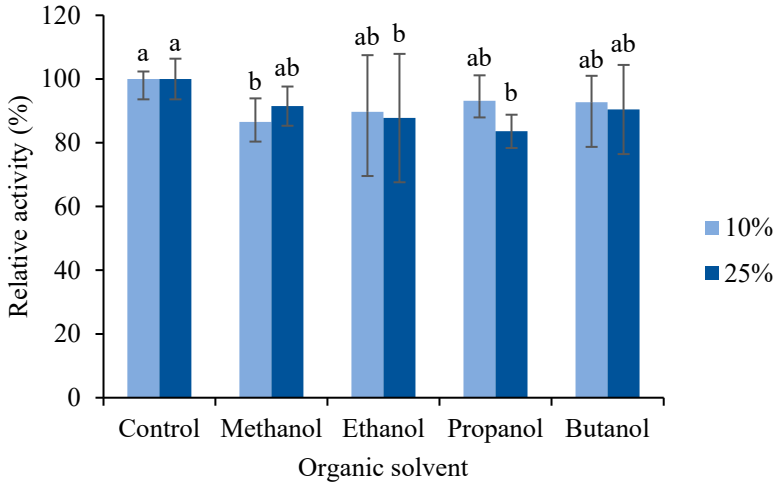

Figure 3. Effect of organic solvents on amylase activity. Different superscripts for each concentration show a significant difference ($p < 0.05$).

3.2.5. Effect of Metal Ion on Amylase Activity

Metal ions activate or inhibit enzymes by involving the catalytic ability or modifying the structural conformation. As shown in Figure 4a, amylase activity was decreased in the presence of Na^+, Ca^{2+}, Mn^{2+}, Mg^{2+}, K^+, and Fe^{3+} at 5 mM of metal concentration. The relative activity of all metal ions retained >75% at 5 mM of metal concentration. According to a previous study, Timilsina et al. [32] reported that α-amylase activity was reduced in the presence of 0.1 to 10 mM of Ba^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, K^+, Mg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Sn^{2+}, and Zn^{2+}.

In contrast, this study showed that the amylase was stable and maintained the activity in the presence of 10 mM of Mn^{2+} and Fe^{3+}. This finding indicated that amylase from *B. subtilis* J12 needs more specific metal ion content and a specific concentration for structural integrity and catalytic activity. Thus, amylase from *B. subtilis* J12 can be identified as a metalloenzyme. Previous studies reported that some metal ions increased amylase activity due to the stabilization of the active conformation. In the presence of metal ions, the active site of the enzyme can be improved by changing the active conformation. For instance, Bano et al. [33] reported that 1 mM of Mn^{2+}, K^+, and Fe^{2+} activate and raise amylase activity by about 363%, 165%, and 147%, respectively. Similarly, Karaca Açarı et al. [34] observed that divalent metal ions like Mn^{2+} and Co^{2+} at 5 mM of concentration have similar increases in free-amylase and immobilized-amylase activity.

3.2.6. Effect of Ferric Ion on Amylase Activity

In the presence of ferric ions, the relative activity was increased at 15 mM concentration up to 132% that of a control sample without ferric ions (Figure 4b). However, higher concentrations of ferric ions decreased the activity and retained about 18% activity at 25 mM ferric ion concentrations. These findings indicated that amylase from *B. subtilis* J12 was a metalloenzyme, since it needed specific ions at an optimum concentration of ferric ions. Ferric ion stabilizes and prolongs amylase activity by improving the active site or combining with enzymes. Thus, ferric ions played a role as a cofactor that increased the catalytic activity. In contrast, if the concentration of ferric ions is iexcessive, they might inhibit enzyme actives sites in binding substrate. Kizhakedathil and C [35] reported that α-amylase activity from *Pseudomonas balearica* VITPS19 increased when 1 mM of Ca^{2+} was added and decreased at greater concentrations.

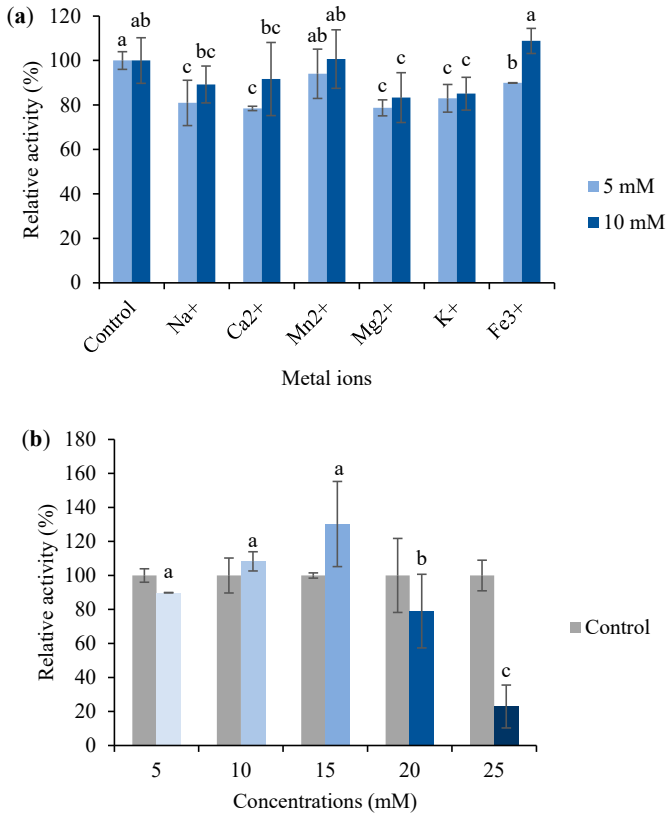

Figure 4. Effect of (**a**) metal ions and (**b**) ferric ions on amylase activity. Different superscripts for each concentration show a significant difference ($p < 0.05$).

3.3. Hydrolysis Pattern of B. subtilis J12 Amylase

A starch hydrolysis pattern from *B. subtilis* J12 crude amylase was observed. As shown in Figure 5, glucose and maltose were the hydrolysis products that are shown in a red square (lane 7). However, starch hydrolysate also showed molecules larger than glucose and maltose, as shown in a blue square (lane 7). It indicated that crude amylase released incompletely hydrolyzed starch. A crude preparation of amylase showed lower degradation of starch than amylase partially purified by ammonium sulfate precipitation [36]. It means the purification step increased the enzyme purity and influenced the hydrolysis results. Thus, the high purity enzyme resulted in more specific products.

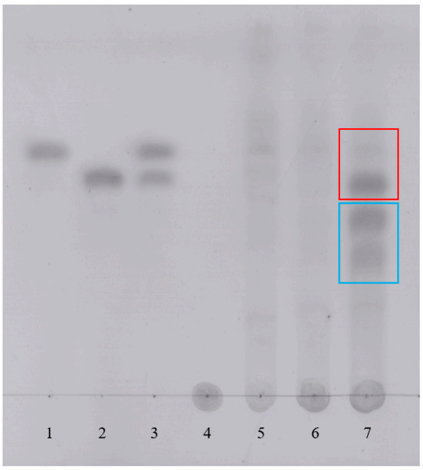

Figure 5. The TLC chromatogram of starch hydrolysate by *B. subtilis* J12 amylase. 1 glucose; 2 maltose; 3 glucose and maltose; 4 1% starch; 5 crude amylase; 6 control; 7 starch hydrolysate; Red square small sugar molecules; blue square large sugar molecules.

3.4. Partial Purification of Amylase

As shown in Figure 6, the peak of protein detection (λ 280 nm) was found in several fractions, while amylase activity was found in fraction 9 (F9) and fraction 10 (F10). These findings indicated that during the washing step with Tris buffer pH 8.0, amylase was still bound to the anion exchanger. Furthermore, amylase was eluted the late phase with NaCl.

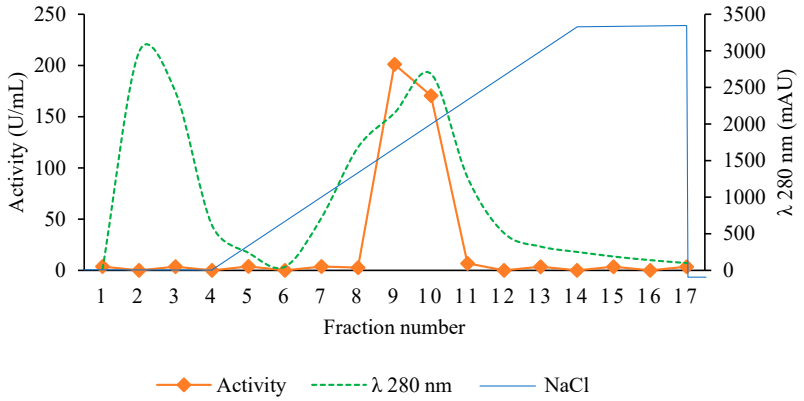

Figure 6. Chromatograms obtained from NGC Quest 10 Chromatography System equipped with the anion-exchange column (Mecro-Prep High Q).

The purity of *B. subtilis* J12 amylase was verified by Native PAGE, SDS-PAGE, and zymography analysis. Several protein bands were found in SDS-PAGE and Native PAGE (Figure 7a,b). When compared with Native zymography, the results in the red square showed that two clear zones appeared on lanes 1 to 4 (Figure 7c). It confirmed the presence of at least two types of *B. subtilis* J12 amylase. However, it could not indicate the molecular weight of the specific enzyme, due to the presence of protein contamination in the partially purified enzyme. Further purification steps to obtain pure amylase should be considered, to determine the type of amylase and increase the enzyme efficacy when it is applied for many purposes. Other studies reported that amylase from *Anoxybacillus ayderensis* had a molecular weight of 58.5 kDa [37] and amylase from *Bacillus* sp. had a molecular weight ranging from 43 to 240 kDa [38].

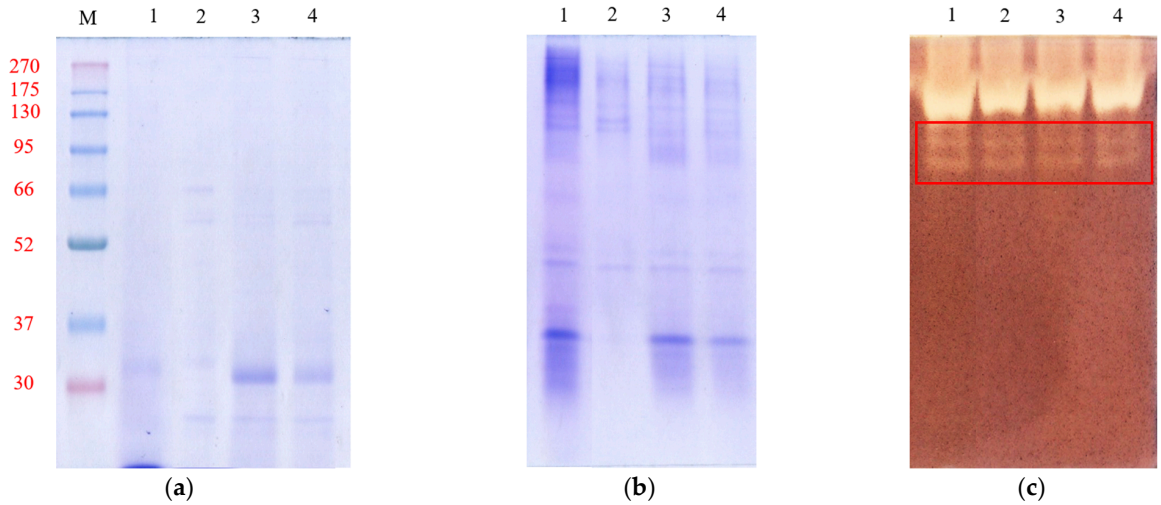

Figure 7. (**a**) SDS-PAGE; (**b**) Native PAGE; (**c**) Native zymography of partially purified amylase from *B. subtilis* J12. M marker 1 crude enzyme; 2 F9; 3 F10; 4 mixed F9 and F10.

3.5. Feasibility of Media Cost for Amylase Production

The media and substrate contribute to a significant portion of the overall expenses for enzyme production. Medium optimization and selection are the first steps to reduce

production costs. The cost for production media for *B. subtilis* J12 amylase was evaluated and compared with other amylase producers that utilize agro-industrial waste (Table 2). In this case, the agro-industrial residue was assumed to be a free by-product to create low-cost media and make equal comparisons among the agro-industrial residues. Compared with other amylase producers, *B. subtilis* J12 used only okara as the media and substrate in amylase production, including the extraction process, without additional ingredients. Consequently, this study concluded that there is no cost forproduction of *B. subtilis* J12 amylase media.

Table 2. Cost comparison of media for amylase production under solid-state fermentation.

Producer	Media Composition *	Material Cost (USD/100 g)	Material Used (g/L of Enzyme)	Cost Per Unit (USD)	Total Cost (USD)	Enzyme Activity	Productivity (U/h)	Reference
B. subtilis J12	Okara	0	143	0	0	983 U/g	40.96	This study
B. amyloliquefaciens	Wheat bran Potato peel KH_2PO_4 $MgSO_4{\cdot}7H_2O$ NaCl $Na_2HPO_4{\cdot}7H_2O$ $NaH_2PO_4{\cdot}H_2O$	0 0 26.85 18.81 12.42 66.12 35.25	125 125 0.5 0.25 0.25 3.89 1.50	0 0 0.13 0.05 0.03 2.57 0.53	3.31	99 U/mL	0.41	[23]
B. subtilis KR1	Wheat bran $MgSO_4$ NaH_2PO_4 K_2HPO_4 Soluble starch Yeast extract $Na_2HPO_4{\cdot}7H_2O$ $NaH_2PO_4{\cdot}H_2O$	0 31.08 42.76 92.96 45.36 78.83 66.12 35.25	100 8.02 7.99 11.60 1 1 0.08 0.03	0 2.49 3.41 10.78 0.45 0.79 0.05 0.01	17.98	82.6 U/gds	1.15	[16]
B. velezensis KB 2216	Moong husk Soybean cake Peptone $MgSO_4$ $KH2PO_4$ Fructose $NaNO_3$	0 0 19.33 31.08 26.85 77.34 25.73	40 20 20 1 3 15 5	0 0 3.87 0.31 0.81 11.60 1.29	17.88	75.78 U/mL	1.05	[1]
B. tequilensis TB5	Rice bran Yeast extract K_2HPO_4 $MgSO_4.7H_2O$ $CaCl_2.2H_2O$ Peptone Beef extract NH_4NO_3 $MgSO_4$ NH_4Cl $(NH_4)_2SO_4$ $MgCl_2$ $CaCl_2$ $FeCl_3$	0 78.83 92.96 38.60 33.17 19.33 229.79 15.47 23.11 15.32 18.52 41.42 122.70 59.49	100 1.39 0.14 0.06 0.03 9 4 2 1 1 1 1 5 1	0 1.10 0.13 0.02 0.01 1.74 9.19 0.31 0.23 0.15 0.18 0.41 6.13 0.59	20.19	37.7 U/mL	0.52	[10]
Aspergillus oryzae	Oil cake KH_2PO_4 NH_4NO_3 NaCl $MgSO_4{\cdot}7H_2O$ Tween 80	0 26.85 15.47 12.42 38.60 16.51	500 0.4 1 0.2 0.2 1	0 0.12 0.15 0.02 0.08 0.16	0.53	10,994.7 U/gds	101.8	[27]
Aspergillus oryzae NRRL695	Soybean husk Flour mill waste KH_2PO_4 $NaNO_3$ $MgSO_4{\cdot}7H_2O$ $CaCl_2{\cdot}2H_2O$ $FeSO_4{\cdot}7H_2O$ $MnSO_4{\cdot}H_2O$ $CoCl_2{\cdot}6H_2O$ $ZnSO_4{\cdot}7H_2O$ K_2HPO_4	0 0 26.85 25.73 38.60 33.17 28.33 35.25 130.88 84.78 92.96	34.65 42.35 3.65 2.31 0.38 0.38 0.006 0.002 0.002 0.001 0.22	0 0 0.98 0.59 0.15 0.13 0.002 0.001 0.003 0.001 0.20	2.06	47,000 U/gds	130.5	[25]

* Agro-industrial residue (assumed to be a free material) and additional ingredients (price based on sigmaaldrich.com; accessed date: 25 June 2024).

Moreover, this innovation can produce high activity and productivity of amylase, and it is cheaper than others. They added other materials besides agro-industrial waste into the production media, and the amount of materials varied, which influenced the high total cost for production. Among bacterial amylase producers, *B. subtilis* J12 is superior, depending on the cost of the media, activity, and productivity. In contrast, *B. subtilis* J12 showed superiority in cost for media and production time if compared with fungal amylase producers. Therefore, the design of a low-cost material for the media and rapid production of amylase production in this study can be considered for *B. subtilis* J12 amylase commercialization.

On the other hand, the effectiveness of the fermentation method toward the media also influenced the enzyme productivity. This study emphasized an easier approach for effective amylase production while using okara as the low-cost medium. Since okara contains high fibers, solid-state fermentation is an appropriate technique to produce enzymes. The comparison in Table 2 also proved that solid-state fermentation performed well in the bioconversion of agro-industrial residue to produce amylase as a value-added and eco-friendly product. Solid-state fermentation decreased the downstream liquid-volume treatment, with less stirring during fermentation [15]. It raises awareness of green manufacturing concepts and promoted the circular economy and sustainable agriculture by reducing agro-industrial waste, liquid residue, and energy usage [15,39]. Consequently, it is profitable. Capital and operational expenses can be lowered, as well as supporting the green revolution and ecological transition.

In analyzing the potential of *B. subtilis* J12 for commercialization, this study is limited to evaluating the production media using okara. However, other important factors should be considered, such as capital, operational cost, procurement cost, distribution, and project profit. Primary scale-up of the pilot experiment should be done in the next investigation, to evaluate advanced production effectiveness, determine the downstream processes, evaluate the system quality, and to carry out a cost analysis. In this step, the selection of a fermenter type could be the key to maximal production results and could reflect the sustainability of scaling up enzyme production. Solid-state fermentation has disadvantages. The fermentation should be maintained and monitored regularly, due to the initial moisture loss from the medium during the fermentation period [13,40,41]. Simple, easy-to-use, and cost-effective fermenters are important in the development of amylase production process [27]. Certain types of fermenters or bioreactors are options, such as a tray, rotating disc, column, fixed bed, packed bed, rotating drum, fluidized bed, air-pressure pulsation, airlift, and immersion bioreactors, mechanically stirred reactors, and even plug flow configurations [13,15]. Therefore, many aspects should be evaluated in advance, to achieve the commercialization goals of *B. subtilis* J12 amylase.

4. Conclusions

In the current study, solid-state fermentation of okara by *B. subtilis* J12 was performed to generate 983 U/g amylase within 24 h. The characteristics of the crude amylase indicated an optimal condition at pH 6.0 and 50 °C, and it was stable in the range of 30 °C to 50 °C for 120 min. The amylase activity was inhibited by organic solventsand could be activated by ferric ions as the metalloenzyme. The purification of amylase resulted in two fractions, and both fractions contained at least two types of amylases. The highlight of this finding provides new insight into the competitiveness of *B. subtilis* J12 amylase produced from an agro-industrial residue, i.e., a low-cost okara medium to generate eco-friendly products. Considering the productivity and its characteristics, *B. subtilis* J12 amylase can potentially be commercialized, has economic value, withenergy-saving potential, for industrial use.

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