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Original article

Coproduction of alkaline protease and xylanase from genetically modified Indonesian local *Bacillus halodurans* CM1 using corncob as an inducing substrate

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ABSTRACT

The production of corn generates a substantial amount of agro-industrial waste, with corncob accounting for a significant portion of this waste. In this study, we focused on utilizing corncob as a carbon source and inducer to simultaneously produce two valuable industrial enzymes, protease, and xylanase, using a recombinant strain of *B. halodurans* CM1. Interestingly, xylan-rich corncob not only enhanced the xylanase activity but also induced protease activity of the modified *B. halodurans* CM1 strain. The effect of corncob concentration on the coproduction of protease and xylanase was investigated. Corncob with 6 % concentration induced protease activity of 1020.7 U/mL and xylanase activity of 502.8 U/mL in a 7 L bioreactor under the condition of 1 vvm aeration, 250 rpm agitation, 37 °C temperature, initial pH 9.0, and 40 h incubation period. The protease produced was an alkalothermophilic enzyme whose highest activity was at pH 12 and 50 °C, and it belonged to a serine protease family. This alkalothermophilic protease's activity to some degree was reduced by Co^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}, and K^{+}, but enhanced by Ca^{2+} and Ni^{2+} (at 5 mM). The protease was stable even under the presence of a 15 % concentration of acetone, DMSO, ethanol, and isopropyl alcohol. The protease activity at 30 °C was not considerably changed by the presence of detergent, indicating excellent potential as a washing detergent additive. According to these findings, corncob has the potential to be a substrate for the coproduction of protease and xylanase, which have a wide range of industrial uses.

1. Introduction

Corn (*Zea mays* L.) is a globally significant grain crop and a crucial commodity in Indonesia, ranking second after rice as a major carbohydrate and protein source. Besides its primary use as a food source, corn is also utilized in various industrial and feed applications (Purwono and Hartono, 2006). In 2020, Indonesia produced an estimated 22.5 million tonnes of corn, while global production reached 1.16 billion tonnes (FAO, https://www.fao.org/faostat/en/). However, corn production generates a substantial amount of agro-industrial waste; it is known that 40 % of the entire grain yield is made up of corncob (Miranda et al., 2018). Despite the abundant amount produced, corncob is usually discarded or burnt without any further utilization (Tang et al., 2016). This practice not only overlooks the economic value of the waste but also contributes to environmental problems associated with disposal and pollution (Hong et al., 2016).

Actually, corncob is a potential raw material for many useful products, from biofuels to mattresses (Koundinya et al., 2023). They include functional chemicals such as kaempferol, p-coumaric acid, or dietary fiber (Ashour et al., 2013; Aniola et al., 2009). One promising application of corncob in bioprocess technology is its use as a substrate in enzyme production. Enzymes, as biocatalysts, find applications across various industries, from technical processes to food, feed, and pharmaceuticals (Fasim et al., 2021). Corncob is rich in xylan content (Barl et al., 1991), making it a perfect yet cost-effective substrate for microbial fermentation medium to produce xylanase.

Xylanase is found in many living cells including a variety of microorganisms, comprising bacteria, fungi, yeast, and algae. These

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microorganisms are widely employed in the production of xylanases. The popular xylanase bacterial genus producer is *Bacillus*—an example of xylanase production in solid-state fermentation using bran wheat as an inducer by *Bacillus sp*. TC-DT13 was reported (Rodrigues et al., 2020). Another example, xylanase production by recombinant *Bacillus subtilis* DB104 using corncob in submerged fermentation was also described (Helianti et al., 2016).

As an essential enzyme in industries, xylanase is primarily employed in the pulp industry for biobleaching, or in the paper industry for waste paper deinking processes. The implementation of enzymes in industrial applications has led to reduced energy and water consumption, as well as improved product characteristics. For instance, in the waste-paper recycling industry, the use of enzymes has resulted in significant savings in toner, electricity, water, and carbon emissions, with a reported reduction of 145 kg of CO_2 equivalent per tonne of processed pulp (Jegannathan and Nielsen, 2013).

Among the widely applied enzymes, protease holds the highest global demand, accounting for over 60 % of the whole demand. Proteases are extensively utilized in industries, for example, leather processing, detergent manufacturing, and animal feed production (Ward, 2011). Of the various protease-producing bacteria, species of *Bacillus* are also the most widely used microorganisms for proteases in the commercial sector (Razzaq et al., 2019). Pilot-scale protease production by *Bacillus* sp. C4 using molasses medium has been tried (Prathumpai et al., 2020); optimal protease production using pine needle biomass was also described (Sing and Bajaj 2017); production of alkaline protease using dairy effluent as a medium also has been conducted (Mahakhan et al., 2023).

Several approaches can be employed to improve the economics of enzyme production, including the utilization of inexpensive media such as agro-industrial waste, optimization of fermentation conditions, and enhancing enzyme secretion through genetic engineering (Motta et al., 2013; Prasad et al., 2014; Singh et al., 2017). The above-mentioned reports are enzyme productions by utilization of cost-effective media; however, unfortunately, the vast majority of the mentioned research did not employ recombinant microorganisms.

We have previously reported the construction of Indonesian local recombinant *Bacillus halodurans* CM1 harboring two protease genes in its cell, one in the chromosome and one in the inserted plasmid, and this recombinant bacterium produced higher protease activity than the wild type (Priyanka et al., 2022). The native of this strain is already known as a xylanase producer (Ulfah et al., 2012; Helianti et al., 2014; Nandyawati et al., 2021). In this report, we use this recombinant *B. halodurans* CM1 to simultaneously produce two valuable industrial enzymes, protease and xylanase, using agro-industrial waste corncob. Our study suggests the coproduction of xylanase and protease by this recombinant *B. halodurans* CM1 using corncob as an inexpensive substrate to cut down the enzyme manufacturing cost and offer cost-effective and sustainable agro-industrial waste disposal.

2. Materials and methods

2.1. Microbial strains and media used

The recombinant *B. halodurans* CM1 strain that was constructed previously (Priyanka et al., 2022) was grown in Erlenmeyer flasks with Horikoshi medium (Horikoshi, 1999) for starter and glycerol stock. For long-term storage, a recombinant bacterial strain with 20 % glycerol in a cryopreservation vial at −80 °C was prepared. As seed culture preparation, the bacterial strain was grown in a modified Horikoshi medium (pH 9) agar or broth containing 1 % peptones, 0.5 % yeast extract, 0.1 % KH_2PO_4, and 0.5 % Na_2CO_3 and supplemented with tetracycline 12.5 μg/mL. The production media used was a modified Mamo medium (Mamo et al., 2006) (pH 9.0) containing 0.5 % peptone, 0.1 % KH_2PO_4, 0.2 % NaCl, 0.01 % $MgSO_4$, 0.01 % $CaCl_2$, 1 % Na_2CO_3, and corncob as substrate. The corncob was obtained from a local plantation, dried, and ground to a size of 70–100 mesh.

2.2. Media selection for protease production

To choose the most suitable medium for protease production, 3 kinds of media were used for protease production. The first media was made based on Mamo et al., 2006, the second one was the same media with 2 % skim milk as a substrate, and the third media was the same one with 4.37 % corncob content. The overnight seed culture was prepared and freshly inoculated into the media and growing, after 24 h the protease activity was assessed.

2.3. Effect of corncob concentration on xylanase and protease activity

To investigate the influence of corncob concentration on protease and xylanase production, submerged fermentations were performed using various concentrations of corncob (0, 1, 3, 4.37, 6, 8, and 10 %) in the production media. A loop of bacterial culture was inoculated to a 50 mL Horikoshi medium with tetracycline in a 250 mL flask and incubated at 37 °C at 150 rpm in a reciprocal shaking incubator (Kuhner, Switzerland) until a 0.8 cell density was reached at 600 nm. Ten percent of this inoculum was then added into 50 mL of production media in 250 mL flasks and incubated for 48 h at 37 °C with 150 rpm agitation. Every 8 h during fermentation, samples were taken and evaluated for microbial growth by viable cell count, enzyme activity, and protein content.

2.4. Enzymes production

Larger scale coproduction of protease-xylanase enzymes was carried out in a 10 L stirred tank bioreactor (Bailun Bio, China) with a 7 L working volume. The bioreactor was equipped with pH and polarographic dissolved oxygen sensors (Hamilton, Switzerland). Seven liters of the production medium containing 4.37 % and 6 % corncob, respectively, were filled into bioreactors and autoclaved. Subsequently, 700 mL of freshly grown inoculum with a cell density of 0.8 was then transferred to the bioreactor. The fermentation was conducted with agitation at 250 rpm and aeration at 1 vvm using a ring sparger. The temperature was kept constant at 37 °C by cooling water being circulated through an internal coil, while the pH was left unregulated. Silicone-based defoamer was injected to minimize foam as needed. For 48 h, samples were collected and assayed at 8 h intervals.

2.5. Protease activity assay

The activity of protease was measured based on the casein digestion approach as reported by Takami et al., 1989 with some modifications. A crude enzyme solution (125 μL) and a 0.6 % casein solution (625 μL) were mixed in 0.1 M phosphate buffer (pH 12), and the enzymatic reaction occurred by incubating the mixture at 50 °C for 10 min. To halt the reaction, 625 μL of trichloroacetic acid (TCA) solution (0.11 M TCA, 0.33 M acetic acid, and 0.22 M sodium acetate) was applied to the reaction, followed by a 10-minute incubation at 50 °C. Then, the mixture was centrifuged for 5 min at 12,000 rpm (10626 × g). After centrifugation, up to 300 μL of supernatant was collected and mixed with 750 μL of 0.55 M sodium carbonate and 150 μL of three-folds-diluted Folin-Ciocalteau reagent before incubating at 50 °C for 10 min. The absorbance of the final mixture was measured at 660 nm using tyrosine as a reference. One unit (U) protease activity was defined as the total amount of enzyme necessary to catalyze the production of 1 μg tyrosine per minute under the assay conditions.

2.6. Xylanase activity assay

Xylanase activity was measured by determining the increase of reducing sugar using the 3,5-dinitrosalysilic acid (DNS) method (Miller, 1959). Fifty microliters of crude enzyme extract were mixed with 450 μL

of 0.5 % beechwood xylan in 50 mM Tris-HCl buffer (pH 9) and the enzymatic reaction occurred by incubating the mixture at 70 °C for 10 min. The reaction was immediately stopped by adding 750 µL of DNS solution (1 % 3,5-dinitrosalysilic acid, 0.2 % phenol, 0.05 % sodium sulfite, 1 % sodium hydroxide, and 20 % potassium sodium tartrate) followed by boiling in a water bath for 5 min. After cooling the mixture to room temperature, 250 mL of distilled water was added and the absorbance value was measured at the wavelength of 540 nm. One unit (U) of xylanase activity was defined as the amount of enzyme needed to release 1 µmol of xylose equivalent per minute under the assay condition.

2.7. Crude protease characterization

The optimal pH for *B. halodurans* CM1 protease was determined between 6.0 and 13.0 at 50 °C; whereas, the optimal temperature for protease activity was determined at optimal pH using different temperature ranges (25–80 °C). To examine the pH stability of the protease, the enzyme was pre-incubated at different pH values (6.0–13.0) for 1 h before assay of activity at 50 °C. Meanwhile, to assess the thermostability, the enzyme was pre-incubated at pH 12 under a range of temperatures between 25 and 50 °C in interval times up to 2 h and sequentially measured for the remaining activity.

The protease activity in the presence of different metal ions (Co^{2+}, Ca^{2+}, Ni^{2+}, Mg^{2+}, Fe^{3+}, Zn^{2+}, and K^{+}) with different concentrations of 1, 5, and 10 mM, respectively, was observed. The protease activity in the presence of organic solvents such as ethanol, acetone, DMSO, methanol, and isopropanol with concentrations of 15, 25, and 50 %, was also examined. The effect of the inhibitor on activity was also observed by assaying the protease activity under the existence of PMSF and EDTA with 1 mM and 5 mM—concentrations, respectively. The effect of detergent on protease was assayed at detergent concentrations of 5 % at 30 °C.

2.8. Wash performance test

We followed the previous method with some modifications (Mahakhan et al., 2023). Small squares of white cotton fabric (7 × 7 cm) were smudged with egg yolk and milk chocolate and air-dried. Then, these cotton clothes were soaked in 100 mL of hard water (TDS, 250 ppm) in 300 mL shake flasks under the following conditions: (a) only hard water, (b) added with protease (500 U/mL), (c) added with detergent (1 mg/mL), (d) added with detergent (1 mg/mL) and protease (500 U/mL). After shaking incubation for 2 h at 250 rpm at room temperature, the clothes were cleaned with water, dried, and visually compared with untreated stained clothes (as a negative control).

2.9. Statistical analysis

The experiments were conducted in three replicates, and the presented data were expressed as the mean value with the standard deviation. Duncan's multiple range test, with a significance level set at $P < 0.05$, and one-way analysis of variance (ANOVA) were used in the analysis (RStudio Team, 2022).

2.10. Economic evaluation

The production cost of protease is estimated using the method described in previous reports (Renganath Rao et al., 2019; Wu et al., 2019). The calculated production cost includes raw material and energy costs. All prices for raw materials and energy are provided by local suppliers. In this study, a production capacity of 1000 L is used as the basis for calculating the cost per unit of protease activity.

3. Results

3.1. Comparison of protease activity in different media

Fig. 1 presents the outcomes of using different substrates for protease production by recombinant *B. halodurans* CM1. Out of the tested substrates, 4.37 % corncob showed a significant effect, resulting in high protease production (913 ± 48.2 U/ml). In comparison, when cultivated in a medium enriched with 2 % skim milk, recombinant *B. halodurans* CM1 produced a lower amount of protease (114 ± 2.2 U/ml). On the other hand, in a medium lacking any additional substrates, the protease yield was much lower, reaching only 35 ± 3.1 U/ml.

3.2. Effect of corncob concentrations

The coproduction of alkaline xylanase and protease of recombinant *B. halodurans* CM1 in submerged fermentations has been conducted. This experiment was carried out for 48 h using corncob with a concentration of 0–10 % w/v. Fig. 2a illustrates a direct correlation between higher substrate concentrations and an elevated level of dissolved protein in the media. Furthermore, the influence of corncob concentration on the growth of recombinant *B. halodurans* CM1 was also observed. The cell number reached its maximum (2.3–2.4 × 10^9 CFU/mL) at corncob concentrations of 6 % and 8 %, and decreased at higher concentrations (Fig. 2b).

Maximum xylanase activity (536.1 ± 14.7 U/mL) was observed after 48 h with an 8 % corncob concentration, which was statistically comparable ($p > 0.05$) to the activity at a 6 % corncob concentration at the same incubation time (Fig. 3a). Additionally, extending the incubation time beyond 24 h at corncob concentrations of 4.37 %, 6 %, 8 %, and 10 % did not significantly increase xylanase production.

The highest protease activity (1135.4 ± 23.1 U/mL) by recombinant *B. halodurans* CM1 was found at 6 % corncob concentration at 40 h of incubation (Fig. 3b). The synthesis of protease remained largely unaffected ($p > 0.05$) with further increments in corncob concentration up to 8 %. However, a slight decrease in protease synthesis was observed with an increase in corncob concentration to 10 %.

3.3. Protease and xylanase coproduction in a 10 L bioreactor

We validated the coproduction of protease and xylanase by recombinant *B. halodurans* CM1 using corncob concentrations of 6 % and 4.37 % in a 10-liter bioreactor with a working volume of 7 L. The secretion of protease and xylanase by recombinant *B. halodurans* CM1 was significantly observed in the exponential phase between 8 and 24 h (Fig. 4).

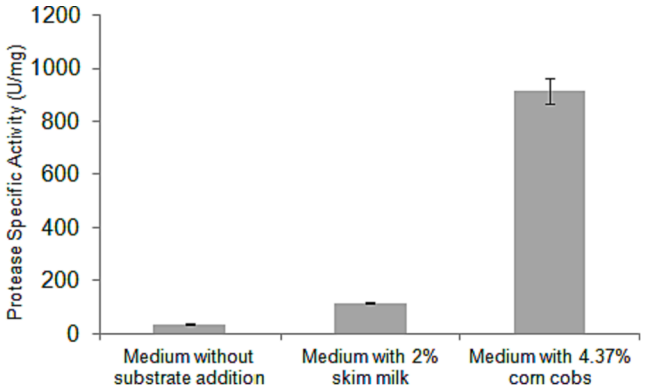

Fig. 1. Protease activity in different mediums: Mamo medium only, Mamo medium with 2 % skim milk, and Mamo medium with corncob 4.37 %. The cultures of recombinant *B. halodurans* CM1 were inoculated at a temperature of 37 °C, pH 9.0, and shaking speed of 150 rpm, and the protease was harvested after 24 h.

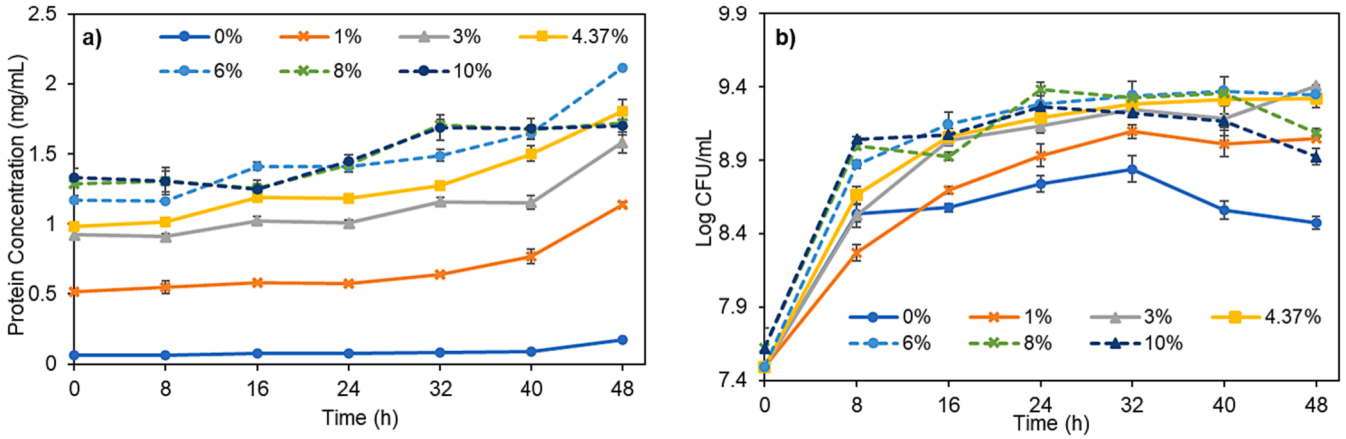

Fig. 2. Effect of corncob concentration on **(a)** soluble protein concentration in the fermentation media and **(b)** cell growth of recombinant *B. halodurans* CM1.

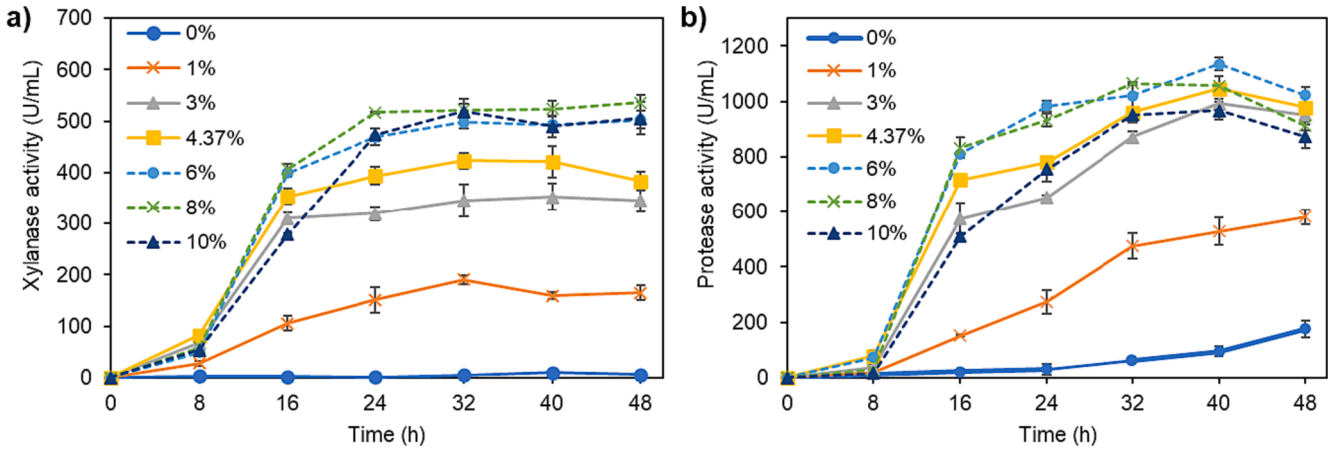

Fig. 3. Effect of corncob concentration on **(a)** xylanase activity and **(b)** protease activity of recombinant *B. halodurans* CM1.

During the final stage of the exponential phase, the highest xylanase activity was recorded as 502.8 ± 23.4 U/mL and 360.8 ± 24.6 U/mL for corncob concentrations of 6 % and 4.37 %, respectively. This activity slightly decreased thereafter. In contrast, the maximum protease activity of 1020.7 ± 26.7 U/mL and 1064.8 ± 56.9 U/mL for corncob concentrations of 6 % and 4.37 %, respectively, was observed during the early stationary phase after 40 h of incubation.

3.4. Characterization of crude protease produced by modified *B. halodurans CM1*

Biochemical characterization of *B. halodurans* CM1 crude protease has been carried out. The protease exhibited significant activity across a broad pH range of 6 to 13 and a wide temperature range of 30 to 80 °C. Remarkably, the protease maintained over 80 % of its activity within the pH range of 11 to 12, with its peak activity observed at pH 12 (Fig. 5a), suggesting that *B. halodurans* CM1 protease functions as an alkaline protease.

The enzyme was highly active at 50–60 °C with the highest proteolytic activity at 50 °C, revealing that this protease was thermo-active (Fig. 5b). Although the enzyme demonstrated its proteolytic activity at 50 °C, it remained notably active at 30 °C. Thermostability studies revealed that the protease was stable at 25–40 °C since it retained 80 % activity after 120 min of incubation (Fig. 5c). In the presence of detergent, the protease activity at 30 °C was not affected significantly, showing its strong suitability for use in washing detergents (Fig. 5d).

Table 1 provides information on how metal ions affect protease activities. The addition of Ca^{2+} (122.2 ± 1.2 %) and Ni^{2+} (117.1 ± 5.5 %) resulted in a modest increase in protease activity. Furthermore, Co^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}, and K^{+} had no considerable impact on protease activity at a concentration of 1 mM. However, at higher concentrations, these metal ions exhibited a partially inhibitory effect.

The protease was quite stable to retain activity above 80 % after 24 h of incubation in organic solvents such as ethanol (80.0 ± 1.6 %), acetone (92.7 ± 1.2 %), DMSO (81.6 ± 3.6 %), and isopropanol (82.5 ± 5.1 %) with concentrations of 25 % (Table 2). This protease was serine protease since it was inhibited significantly by the presence of PMSF (11 ± 2.7 %) but not affected by EDTA (98 ± 0.0 %) (Table 3).

3.5. Washing performance of recombinant *B. halodurans CM1 protease*

The washing performance of *B. halodurans* CM1 protease in hard water was tested on pieces of cloth stained with egg yolk and milk chocolate. This test revealed that protease supplementation in detergents improved washing performance as indicated by better removal of egg yolk and milk chocolate stains than using detergent alone (Fig. 6). The stain removal performance by protease alone is also comparable to that of detergent alone even in hard water.

3.6. Economic evaluation

The economic feasibility of coproducing protease and xylanase by recombinant *B. halodurans* CM1 was evaluated based on the production costs required to produce 1000 L of crude enzymes. Production costs encompass raw material and energy costs, as detailed in Table 4. Raw material costs for media enriched with 6 % corncob and 2 % skim milk

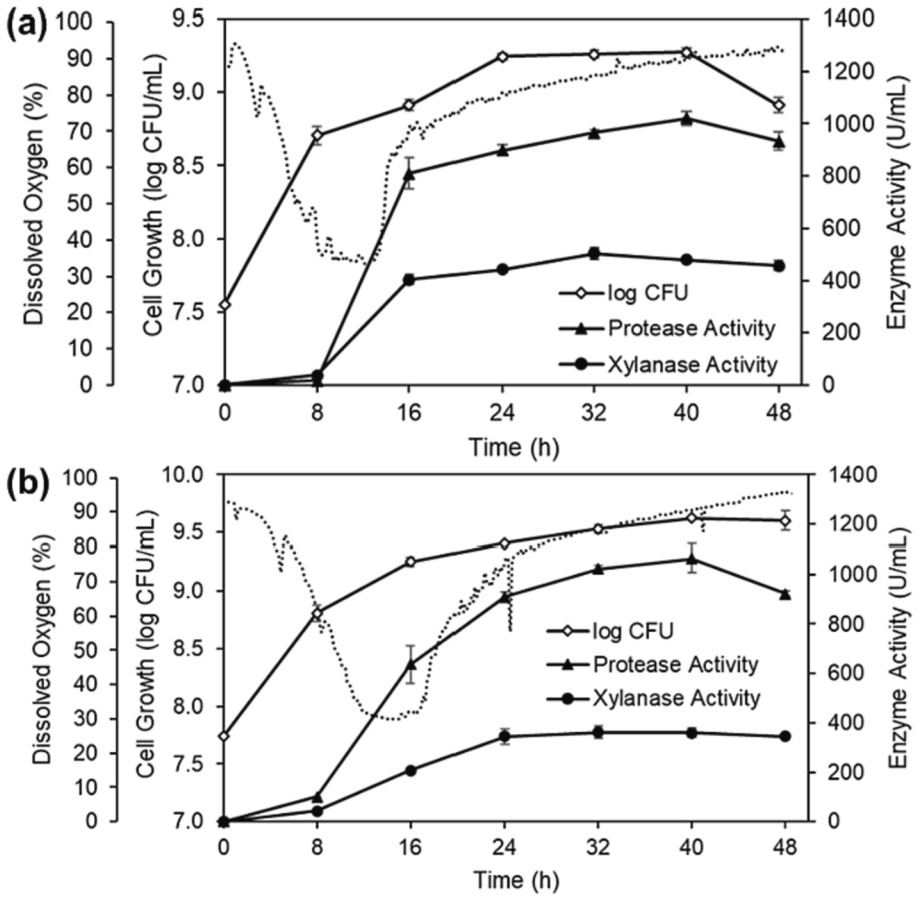

Fig. 4. Profile of dissolved oxygen (dotted line), cell growth (open rhombus), protease activity (filled triangle), and xylanase activity (filled circle) in bioreactor culture at 250 rpm and 37 °C using corncob with the concentration of **(a)** 6 % and **(b)** 4.37 % (w/v).

were US$ 215.01 and US$ 267.24 per 1000 L of crude enzyme, respectively. Economic analysis indicates that using 6 % corncob resulted in a lower fermentation cost (US$ 221.24 per 1000 L of crude enzyme) compared to 2 % skim milk (US$ 273.26 per 1000 L of crude enzyme). Regarding enzyme price per unit of protease activity, media with 6 % corncob yielded a price of US$ 0.24 per 10^6 U, while media with 2 % skim milk yielded a price of US$ 2.40 per 10^6 U.

4. Discussion

Bacillus halodurans strains are recognized for their proficiency in producing alkaline industrial enzymes. Numerous enzymes derived from *B. halodurans* have been documented for diverse industrial applications (Annamalai et al., 2013a; Balachandran et al., 2021; Kaewsalud et al., 2021; Tekin et al., 2021). One specific strain of interest is *B. halodurans* CM1 (NCBI accession number JN903769), an Indonesian local strain that was isolated and characterized previously (Ulfah et al., 2012). This bacterial strain is capable of thriving between 30 and 55 °C at an alkaline pH, with the most favourable growth occurring at 50 °C. Additionally, it produces several industrially important enzymes. *B. halodurans* CM1 strain has undergone genetic modification through the insertion of a recombinant plasmid containing an alkaline protease gene via conjugational transformation. As a result, the bacterial strain possesses two alkaline protease genes in both the plasmid and chromosome. It exhibited tetracycline resistance and demonstrated enhanced protease activity of 893 U/mL in comparison to the wild-type strain's protease activity of 671 U/mL after 24 h of fermentation (Priyanka et al., 2022).

The production and characterization of xylanase using the wild-type bacterial strain have been conducted elsewhere (Nandyawati et al., 2021). However, the protease production simultaneously with xylanase production, especially from genetically modified *B. halodurans* CM1 has not been described. The previous report clearly established the effect of corncob on xylanase production, determining that a 4.37 % concentration was optimal for wild-type *B. halodurans* CM1 (Helianti et al., 2018b). Interestingly, we discovered that the Mamo medium with corncob not only induces xylanase production but also induces protease production. Thus, it is intriguing to explore the coproduction of protease and xylanase using the modified *B. halodurans* CM1 with corncob as the substrate.

Mamo medium contains $CaCl_2$ compared to other alkaliphile mediums reported by Horikoshi (Mamo et al., 2006; Horikoshi, 1999). Ca^{2+} ion has been reported to increase protease and xylanase production through activation and stabilize them (Atalla and Gamal, 2020; Mothe and Sultanpuram, 2016).

The expense associated with production media forms a substantial portion of the total cost involved in enzyme production (Al-Dhabi et al., 2020). Due to its high cost, xylan has been discouraged as a substrate for xylanase production. Since corncob provides xylan and other nutrients required for the growth of microorganisms, it can be utilized as a xylan substitute in xylanase synthesis (Fasiku et al., 2023). In this study, the addition of corncob as a substrate not only induced the production of xylanase but also protease. *B. halodurans* CM1 was able to produce enzymes using this substrate. This might be due to the intrinsic soluble proteins from corncob which can induce protease secretion by microorganisms (de Castro and Sato, 2013). According to several reports, corncob generally contains 41.4 % hemicellulose (xylan), 40 % cellulose, 5.8 % lignin, 2 % crude protein, and 2.1 % starch (Kanengoni et al., 2015; Castorina et al., 2023). The autoclaving process in the sterilization of the production medium probably made some of the nutrients in the

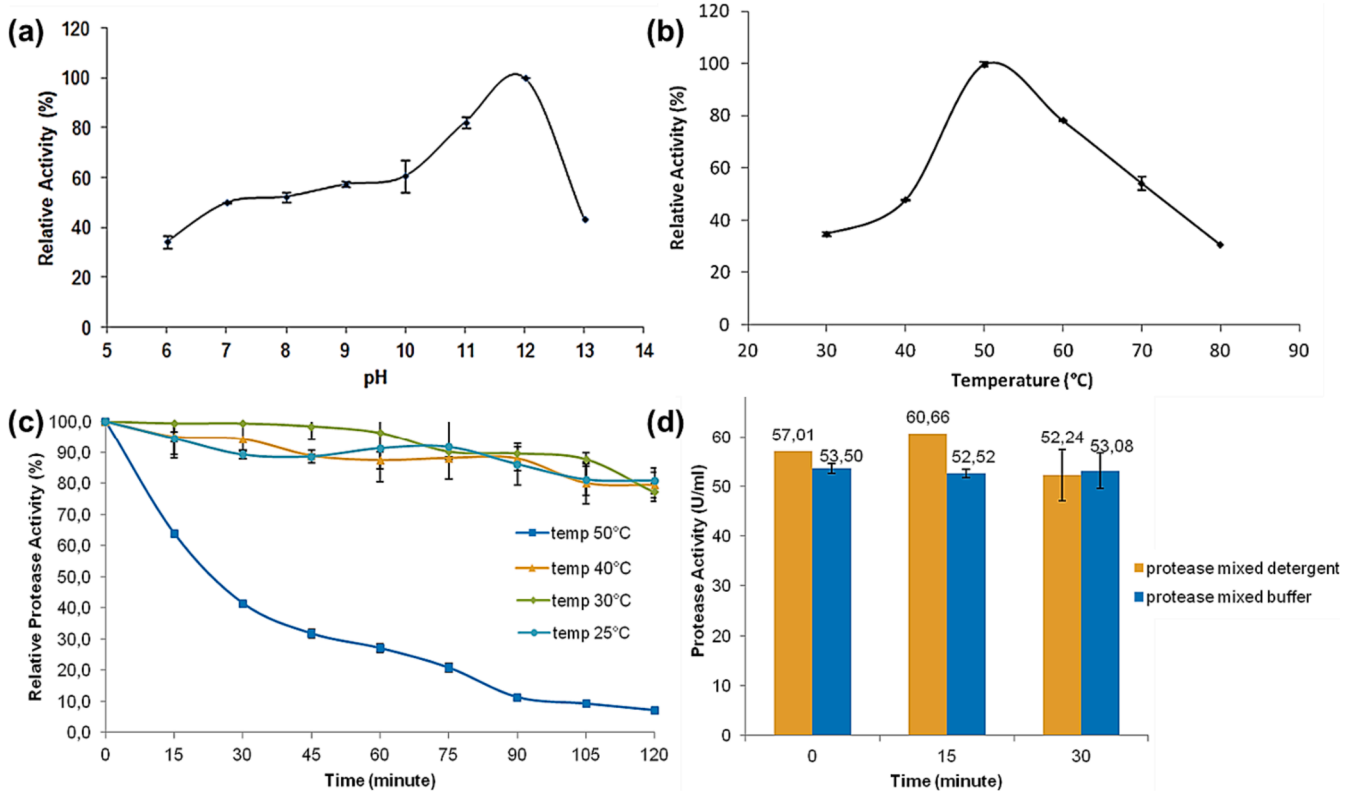

Fig. 5. Characterization of recombinant *B. halodurans* CM1 protease. (**a**) The effects of pH on protease activity. (**b**) The effects of temperature on protease activity and (**c**) stability. (**d**) The effects of detergent on protease activity.

Table 1
The effect of metal ions on *B. halodurans* CM1 protease activity.

Metal Ions	Concentration (mM)	Residual activity (%)
Control	0	100
$CoCl_2$	1	99.39 ± 4.71
	5	83.27 ± 3.22
	10	70.82 ± 0.50
$MgSO_4$	1	97.29 ± 3.72
	5	85.72 ± 2.73
	10	78.36 ± 2.23
$FeCl_3$	1	95.19 ± 1.74
	5	87.65 ± 0.50
	10	74.15 ± 3.72
$CaCl_2$	1	122.18 ± 1.24
	5	119.73 ± 0.25
	10	115.70 ± 2.48
$NiSO_4$	1	117.10 ± 5.45
	5	109.21 ± 2.23
	10	69.07 ± 2.97
$ZnCl_2$	1	94.49 ± 2.73
	5	89.05 ± 5.45
	10	79.59 ± 0.99
KCl	1	98.52 ± 3.97
	5	90.45 ± 5.95
	10	86.77 ± 2.23

Table 2
The effect of organic solvents on *B. halodurans* CM1 protease activity.

Organic Solvents	Concentration (%)	Relative activity (%) (1 h)	Relative activity (%) (24 h)
Control	0	100	100
Methanol	15	91.92 ± 5.42	69.35 ± 2.94
	25	83.05 ± 2.79	62.12 ± 4.49
	50	43.47 ± 0.00	38.39 ± 2.48
Ethanol	15	91.05 ± 0.15	93.24 ± 2.01
	25	81.29 ± 0.93	79.98 ± 1.55
	50	43.82 ± 0.00	52.41 ± 2.73
Acetone	15	96.09 ± 0.15	95.32 ± 4.03
	25	81.95 ± 2.17	92.69 ± 1.24
	50	39.62 ± 0.00	58.73 ± 3.97
DMSO	15	96.63 ± 3.10	91.16 ± 1.55
	25	82.61 ± 4.96	81.62 ± 3.56
	50	53.47 ± 0.00	51.19 ± 2.73
Isopropanol	15	96.09 ± 4.80	91.05 ± 2.01
	25	81.08 ± 5.58	82.50 ± 5.11
	50	43.12 ± 0.00	49.87 ± 0.12

Table 3
The effect of protease inhibitors on *B. halodurans* CM1 protease activity.

Inhibitor	Concentration	Residual Protease Activity (%)
None	–	100 ± 0.0
PMSF	1 mM	11 ± 2.7
EDTA	10 mM	98 ± 0.0

corncob easily released into the medium.

Maximum xylanase activity was obtained with 8 % corncob after 48 h of incubation. Higher corncob concentrations in our study resulted in decreased xylanase activity, possibly due to the presence of excess xylose in the fermentation media (Gupta and Kar, 2009) and inadequate mixing at higher substrate concentrations (Adhyaru et al., 2014). The decreased xylanase activity at a corncob concentration of 10 % might also be due to the decrease in bacterial growth (Fig. 2b). Previous studies have reported that *Bacillus* is less effective in utilizing xylose, and higher certain xylose concentration could inhibit *Bacillus* cell growth, consequently leading to a decrease in target enzyme activity (Thi Nguyen and Tran, 2018; Zhu and Sui, 2018). These results align with the study by Helianti et al., 2016 which reported that the addition of corncob at concentrations above 4 % led to reduced cell growth and xylanase

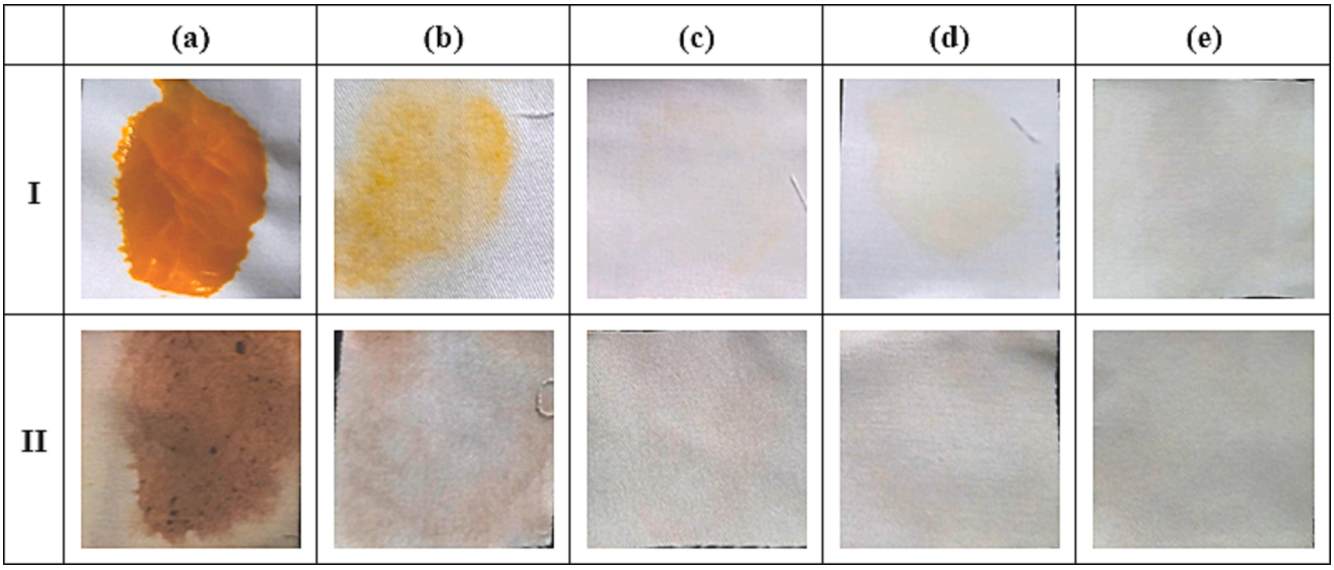

Fig. 6. Washing performance analysis of *B. halodurans* CM1 protease. Pieces of cloth stained with **(I)** egg yolk and **(II)** milk chocolate. **(a)** Control (untreated stained cloth) and stained cloth washed with **(b)** hard water, **(c)** *B. halodurans* CM1 protease (500 U/mL), **(d)** detergent (1 mg/mL), and **(e)** detergent (1 mg/mL) + *B. halodurans* CM1 protease (500 U/mL).

Table 4
Cost estimation of protease and xylanase coproduction by recombinant *B. halodurans* CM1.

	Mamo + 6 % corncob	Mamo + 2 % skim milk	Unit Cost
Working volume	1000 L	1000 L	
Protease activity	913 U/mL	114 U/mL	
Seed culture preparation			
Peptone	1.11 kg	1.11 kg	12.74 US $/kg
Yeast extract	0.55 kg	0.55 kg	114.65 US $/kg
KH_2PO_4 p.a. grade	0.11 kg	0.11 kg	63.69 US $/kg
Na_2CO_3 p.a. grade	0.55 kg	0.55 kg	70.06 US $/kg
Enzyme production			
Peptone	5 kg	5 kg	2.50 US$/kg
KH_2PO_4	1 kg	1 kg	5.10 US$/kg
NaCl	2 kg	2 kg	0.64 US$/kg
$MgSO_4$	0.1 kg	0.1 kg	1.02 US$/kg
$CaCl_2$	0.1 kg	0.1 kg	0.96 US$/kg
Na_2CO_3	10 kg	10 kg	0.96 US$/kg
Corncob	60 kg	0 kg	0.19 US$/kg
Skim milk	0 kg	20 kg	3.18 US$/kg
Feedstock cost	US$ 215.01	US$ 267.24	
Steam consumption	246.9 kg	238.8 kg	0.02 US$/kg
Steam cost	US$ 4.94	US$ 4.78	
Electricity consumption	18.2 kWh	17.5 kWh	0.07 US $/kWh
Electricity cost	US$ 1.29	US$ 1.24	
Total production cost per 1000 L	US$ 221.24	US$ 273.26	
Production cost per 10^6 U of protease	US$ 0.24	US$ 2.40	

production in recombinant *B. subtilis* DB104.

Recombinant *B. halodurans* CM1 achieved its maximum protease activity at a corncob concentration of 6 %. The lower protease activity at higher corncob concentrations may be related to lower cell viability at these higher concentrations (Fig. 2b). There was no significant increase in cell viability when the corncob concentration increased from 6 % to 8 %. Cell viability even decreased when corncob concentration was raised to 10 %. Similar profiles of protease activity, mirroring the cell viability profiles, are shown (Fig. 3b). Additionally, the decrease in protease activity may also attributed to the presence of specific amino acids from corncob. Although the presence of proteins and amino acids up to a certain level could increase the protease activity, at a certain level, amino acids from corncob could lead to a decrease in protease production as well as *Bacillus* cell growth (Meißner et al., 2022; Lau et al., 2019; Sharma and Singh, 2016). Several studies found that the synthesis of extracellular protease was suppressed at high substrate concentrations. A study by Sharma and Singh, 2016, suggested that protease synthesis by *Nocardiopsis dassonvillei* OK-18 was repressed by certain amino acids. Ibrahim et al., 2015 reported that an increase in yeast extract concentration above 2 % significantly reduced protease production by *Bacillus* sp. NPST-AK15, but did not affect bacterial growth. In another study, Fakhfakh et al., 2011 found that increasing the substrate concentration to 7 % decreased the production of keratinolytic proteases by *B. pumilis* A1.

Although the production of protease by the recombinant *B. halodurans* CM1 strain appears to be growth-associated, a previous study by Priyanka et al., 2022 showed that the increased protease activity in the recombinant *B. halodurans* CM1 was attributed to the expression of two protease genes within *B. halodurans* CM1 cells. Additionally, despite several studies showing proteolytic degradation of other enzymes during the simultaneous production of protease, this study found that xylanase remained stable in the presence of highly active protease. Interestingly, there was no significant decline in xylanase activity during the late stationary phase. Furthermore, the xylanase activity remained stable for up to 48 h, whereas the protease activity started to decrease after 40 h of fermentation.

This study suggests the use of corncob at a maximum of 6 % to be scaled up to the bioreactor because increasing corncob concentration did not provide a significant increase in protease and xylanase activity. Therefore, the use of corncob with a concentration of 6 % will be more economical and energy efficient. In addition, the high loading of solid substrates will increase the viscosity of the medium, thereby complicating the operation of the bioreactor and the subsequent recovery process (Garai and Kumar, 2013; Moteshafi et al., 2019).

The coproduction experiments conducted at the bioreactor scale demonstrated that the peak protease activity was achieved during the initial stationary phase. This is consistent with previous studies which also reported several alkalophilic bacteria producing maximum proteases at the beginning of the stationary phase (Anandharaj et al., 2016; Annamalai et al., 2013b; Maruthiah et al., 2017; Tuysuz et al., 2020).

The consistent results obtained in the bioreactor and the flask demonstrated the potential of utilizing corncob in a larger submerged fermentation system to produce enzymes simultaneously. In addition, a comparison of the coproduction of xylanase and alkaline protease under submerged fermentation conditions shows that the xylanase and protease obtained in this study are higher than those produced by *B. licheniformis* using wheat bran as a substrate (5.49 U/mL xylanase and 4.87 U/mL protease) which was conducted in a 50 mL production medium (Limkar et al., 2019).

The characterization of xylanase produced by this strain was described elsewhere (Helianti et al., 2018b; Nandyawati et al., 2021) which is the native xylanase; and its recombinant xylanase has the potential to be used for the deinking of waste paper (Helianti et al., 2018a; 2014). In this study, biochemical characterization of the enzyme showed its protease activity over a wide pH range with maximum activity at pH 12. Moreover, the enzyme demonstrated its stability when exposed to 5 % commercially available detergents. These findings indicate that *B. halodurans* CM1 protease holds promise for application in alkaline environments, specifically as an additive in the detergent industry. Washing performance tests confirm the effectiveness of *B. halodurans* CM1 protease as a potential additive for use in detergent formulation. In addition, its stability in the presence of Ca^{2+} in concentrations up to 5 mM indicates its suitability for laundry detergents for washing in hard water in which calcium ions are present. These observations are consistent with prior studies on alkaline proteases derived from *Bacillus* spp., which was stimulated in an environment with Ca^{2+} ions (Balachandran et al., 2021; Daoud et al., 2018; Joshi and Satyanarayana, 2013; Saggu and Mishra, 2017; Vijayaraghavan et al., 2014).

The thermo-activity and thermostability of the enzyme demonstrate promising potential for utilization in the detergent industry, where enzymes are commonly formulated for incorporation into detergents. The thermostable nature of the enzyme ensures stability during spray drying (Gurumallesh et al., 2019). Additionally, the thermostable properties of this enzyme hold promise as a detergent additive, especially considering that some fabrics such as cotton are washed at high temperatures (Vojcic et al., 2015). Similar thermo-activity and thermostability are demonstrated by *Pyxidicoccus* sp. 252 alkaline protease, which has maximum activity between 40 and 50 °C and remains stable at 50 °C (Sharma et al., 2021). Furthermore, its ability to maintain stability in the presence of ethanol, DMSO, acetone, and isopropanol indicates its potential application in the synthesis of enzymatic products involving organic solvents. These results are similar to the alkaline protease from *Stenotrophomonas maltophilia* strain SK, which exhibits resistance to ethanol, methanol, isopropanol, and acetone (Waghmare et al., 2015).

The production of proteases with biochemical properties similar to those of recombinant *B. halodurans* CM1 has also been reported previously, e.g., *B. infantis* SKS1 produced 375 U/mL protease activity (Saggu and Mishra, 2017), *B. halodurans* US193 produced 505 U/mL protease activity (Daoud et al., 2018), *B. licheniformis* K-3 produced 1321 U/mL protease activity (pine needle biomass as substrate) (Singh and Bajaj, 2017), and *Pyxidicoccus* sp. 252 produced 3000 U/mL protease activity after 120 h incubation (Sharma et al., 2021). The coproduction of protease and other enzymes for detergent application was also reported in a previous report. *Bacillus nealsonii* PN-11 coproduced thermo-alkalistable mannanase and protease active in wide temperature and pH ranges. Both enzymes were applied in detergent. The coproduction was conducted with wheat bran as substrate and the activity was less than the enzyme produced in this study (David et al., 2018). However, the microbial strains were wild-type ones, and the substrates used were different. Furthermore, the research only described protease, not the enzyme coproduction. Our research is extremely relevant to the local agriculture and environmental conditions.

The successful scale-up of protease and xylanase coproduction from flask to bioreactor indicates the technical feasibility of scaling up to larger volumes. To gain insights into the economic feasibility of coproducing protease and xylanase using corncob, a production cost calculation was conducted, similar to the approach taken by Wu et al., 2019 and Renganath Rao et al., 2019. Their approach involved calculating the expenses for raw materials and energy to determine the production cost per unit of enzyme. In this study, we adopted a similar methodology, calculating production costs based on a 1000 L enzyme production capacity. Under the same fermentation conditions, utilizing 6 % corncob as a substrate resulted in a cost reduction compared to using 2 % skim milk. Despite the increased energy consumption associated with the use of 6 % corncob, raw material costs decreased by up to 20 %. The higher protease activity in the 6 % corncob medium resulted in significantly lower production costs per unit of protease activity (US$ 0.24 per 10^6 U of protease), up to ten times less than the production costs using 2 % skim milk (US$ 2.40 per 10^6 U of protease). For comparison, Renganath Rao et al., 2019 reported a production cost of US$ 0.44 per 10^6 U for alkaline protease from *Brevibacterium luteolum* (MTCC 5982), utilizing bran as the substrate for solid-state fermentation. These findings highlight the cost-effectiveness of utilizing corncob as a substrate for protease and xylanase production.

5. Conclusions

This work demonstrated the single step of coproduction of alkaline xylanase and protease of recombinant *B. halodurans* CM1 using agriculture waste corncob. The addition of corncob induced the production both of xylanase and protease with the maximum concentration for xylanase and protease of 502.8 U/mL and 1020.7 U/mL, respectively. The results show that corncob was an economically excellent substrate for these two enzymes' production. The findings of this study hold significance in terms of optimizing parameters for future scale-up investigations at the bioreactor level. Additionally, they provide insights into the economic viability of this enzyme coproduction process.

CRediT authorship contribution statement

I Gede Eka Perdana Putra: Writing – original draft, Writing – review & editing, Validation, Methodology, Investigation. **Maria Ulfah:** Validation, Methodology. **Niknik Nurhayati:** Validation, Methodology. **Is Helianti:** Conceptualization, Funding acquisition, Data curation, Writing – original draft, Writing – review & editing, Validation, Methodology, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contributions

I Gede Eka Perdana Putra: conducted the coproduction from the Erlenmeyer flask scale to the bioreactor scale, performed the assay, and some characterization, and wrote the first draft of the manuscript. Maria Ulfah: conducted the biochemical characterization assay. Niknik Nurhayati: conducted the biochemical characterization assay. Is Helianti: corresponding author, responsible for the overall research, designed and supervised the overall experiments, corrected and approved the final manuscript. I Gede Eka Perdana Putra and Is Helianti are the main contributors. All authors read and approved the final manuscript.

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