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Contents

Review

Agricultural Biotechnology


Biocatalysis and Bioprocess

Bioactive Agents

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Contents

Review

Agricultural Biotechnology

Biocatalysis and Bioprocess

Bioactive Agents

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Distribution, purification and characterization of a monofunctional catalase from *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae)


Manal M.E. Ghanem, Magda A. Mohamed, Ahmed M. Abd-Elaziz
Article 101480


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Evaluating differential effect of deltamethrin and carbofuran on growth characteristics of *Westiellopsis prolifica* Janet, a dominant nitrogen fixing cyanobacterium of tropical rice field ecosystem


Jyotishmita Dutta, P.P. Baruah
Article 101490


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 Export citations

Show all article previews

Contents

Review

Agricultural Biotechnology

Biocatalysis and Bioprocess

Bioactive Agents

Lignocellulases : from Isolation to Structure-Function Mechanism and Industrial Application; edited by Ni Nyoman Tri Puspaningsih, Kazuo Sakka, Muhammad Mukram Mahamed Mackeen and Rosli Md Illias

Research article • Full text access

Heterologous expression and characterization of thermostable lipase (Lk1) in *Pichia pastoris* GS115

Baiq Repika Nurul Furqan, Akhmaloka
Article 101448


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Almando Gerald, Ni'matuzahroh, Fatimah, Chang-Hao Cui, ... Sun Chang Kim
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Contents

Review

Agricultural Biotechnology

Biocatalysis and Bioprocess

Bioactive Agents


Industrial Enzymes

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Contents

Review

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Biocatalysis and Bioprocess

Bioactive Agents


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Mohamad Farizuan Abdullah, Jamaliah Md Jahim, Peer Mohamed Abdul, Safa Senan Mahmod


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Lactobacillus casei fermentation towards xylooligosaccharide (XOS) obtained from coffee peel enzymatic hydrolysate

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ABSTRACT

Coffee peel waste contains fibers including xylan, a polymer that constitutes hemicelluloses. Xylan can be extracted from coffee peel and used for xylooligosaccharide (XOS) production. XOS is an oligomer that has the potentials as a prebiotic. XOS production could be carried out by enzymatic hydrolysis using endo- β -1,4-D-xylanase. In this research, the XOS from coffee peel was investigated for its prebiotic potential on the growth of *Lactobacillus casei* (*L. casei*) in-vitro. The assay was carried out by adding various concentrations of XOS to the bacterial culture media under different incubation times. *L. casei* growth was calculated based on the formed bacterial colonies log CFU/mL. Addition of 20% XOS to 10 mL of *L. casei* growth media showed the best result at 12 h with 8.75 log CFU/mL. Increased bacterial colonies proved that XOS in fermented media was beneficial for the bacteria, resulting in bacterial cell proliferation. Other parameters observed in this study were the decreased pH value, the reduced XOS level and the increased content of organic acids in the bacterial culture media.

1. Introduction

Prebiotics are non-living food components beneficial to probiotic bacteria because they modulate microbiota (FAO, 2007). Prebiotics mostly come from oligosaccharides, a carbohydrate comprising 3–10 monosaccharide units (Roberfroid, 2007).

Widely known prebiotics are inulin, fructooligosaccharides, and galactooligosaccharides (Roberfroid, 2007; Kelly, 2009). Other carbohydrates that have potential as prebiotics are glucooligosaccharide, lactulose, lactosucrose, palatinose and XOS (Roberfroid et al., 2010). XOS is a promising prebiotic because it can be obtained from cheap and renewable agricultural wastes (Samanta et al., 2015).

XOS are oligomers of xylan arranged in β -(1,4)-xyloside bonds. XOS are obtained from the hydrolysis of xylan that can be found in many plants (Brienzo et al., 2010). XOS has been produced from various sources of agricultural waste such as corn hull, garlic bagasse and cassava peel (Vazquez et al., 2000; Moure et al., 2006; Samanta et al.,

2012; Kallel et al., 2014; Hafidah et al., 2018). Coffee peel waste contains a reported 85% fiber, composed mainly of cellulose and hemicellulose (Borrelli et al., 2004). Hemicellulose is a heteropolysaccharide that forms a plant cell, with xylan as its constituent polymer. Many types of research have been pursued about ways to add value of the waste of the coffee industry, such as the utilization spent coffee grounds (SCG), coffee pulp (CP), coffee husk (CH), and coffee silver peel (CSS) as a resource biodiesel, fuel pellets, ethanol, adsorbents, and antioxidants, biogas (Kondamudi et al., 2008; Franca et al., 2009; Ramalakshmi et al., 2009; Murthy and Naidu, 2012; Fan et al., 2001; Machado et al., 2002; Jayachandra et al., 2011). In previous research, we have succeeded in getting XOS from the xylan hydrolysis process from all the part of the coffee peel waste, considering the separation of waste coffee is not economical (Unpublished).

XOS production can be carried out through autohydrolysis or enzymatic methods. Enzymatic XOS production is preferred because it is specific and will produce less by-products (Jain et al., 2015). XOS

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compounds obtained from cassava peel was produced by enzymatic hydrolysis using endo- β -1,4-D-xylanase and the compound were mainly obtained in the form of xylopentaosa (Ratnadewi et al., 2016). Endo- β -1,4-D-xylanase enzyme can specifically break the β -1,4 xylo-side bonds of the xylan (Liab et al., 2000). Endo- β -1,4-D-xylanase catalyzes the hydrolysis reaction on β -1,4-xyloside bonds of the main xylan chain thus producing a simple oligomer in the form of XOS. Almost all endoxylanases could hydrolyze the main chain of xylan which are not substituted by the arabinose group (Tuohy et al., 2001).

Clinical studies have proved that the presence of *Lactobacillus* and *Bifidobacterium* in the intestine could inhibit pathogenic bacteria, could increase body immunity, and produces anticarcinogenic effects (Sullivan and Nord, 2002). XOS stimulated the growth of several probiotic strains in-vitro such as *B. adolescentis* NDRI 236, *B. bifidum* NCDC 2715, *B. bifidum* ATCC 29521, *L. brevis*, NDRI *L. plantarum* 184 after 48 h of incubation (Manisseri and Gudipati, 2012). XOS from corn hull is known to increase the growth of *Enterococcus faecium*, *Enterococcus faecalis*, *L. maltromicus*, *L. viridiscens* (Samanta et al., 2012). In line with the in-vitro XOS fermentation profile, XOS was also known to selectively stimulate the growth of intestinal microflora in-vivo. The administration of XOS in mice significantly increased the faecal water content, total cecum weight and *Bifidobacteria* population which decreased the caecum pH value (Chung et al., 2002; Chan et al., 2005). Healthy and diabetic mice fed with 5% or 10% XOS showed a significant increase in the population of *Bifidobacteria* and cecum lactobacilli (Gobinath et al., 2010). Increased populations of beneficial intestinal microflora were accompanied by several changes, including decreased caecum pH value and increase the total weight of cecum. Decrease in pH value was associated with the increase of short-chain fatty acids from selective prebiotic fermentation by intestinal microflora such as *Bifidobacteria* and *Lactobacilli* (Gibson and Roberfroid, 1995).

The aim of this study is to investigate the ability of XOS, produced by enzymatic hydrolysis with endo- β -1,4-D-xylanase from coffee peel, in increasing the growth of *L. casei* in-vitro. The assay was carried out by adding XOS from coffee peel in *L. casei* modified bacterial culture media.

2. Materials and methods

2.1. Materials

Coffee peel (Robusta) was grounded and sieved through a sieve (200 mesh). The powder was extracted for its xylan content, then stored in tightly closed containers at room temperature. *Bacillus sp.* from termites were used for producing endo- β -1,4-D-xylanase enzymes and stored in glycerol stock (-20°C) (Ratnadewi et al., 2016). XOS (X1-X6) (Merck and Megazyme) was as the standard for characterizing the hydrolysis result.

2.2. Enzymatic hydrolysis of coffee xylan and analysis of XOS

Coffee peel xylan solution 0.8% (w/v) was used as the substrate, then endo- β -1,4-D-xylanase enzyme was added at a ratio of 1:1. The mixture was incubated at 40°C for 24 h. XOS was then centrifuged and filtered with $0.2\ \mu\text{m}$ (millipore) filter syringe prior to its constituent types analysis with Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC).

2.3. Fermentation of XOS with *L. casei*

MRSB-m media (modified MRSB) was made by mixing 1 g tryptophan, 0.8 g meat extract, 0.4 g yeast extract, 0.2 g K_2HPO_4 , 0.1 g Tween 80, 0.2 g sodium acetate, 0.02 g magnesium sulfate, 0.004 g manganese sulfate, and 0.2 g $(\text{NH}_4)_2\text{CO}_3$. The mixture was dissolved

with distilled water to a volume of 100 mL. The solution was homogenized and added with XOS from coffee peel solution (Table 1), and then sterilized. The *L. casei* pre-cultured at 37°C for 16 h was added in MRSB-m according to Table 1. Incubation was then carried out aerobically at 37°C for 12, 24, 36 and 48 h without shaking.

2.4. Calculation of lactic acid bacterial populations (BAM method, 2001)

One mL of fermented sample was added to 9 mL of saline solution, followed by serial dilutions (10^{-1} - 10^{-8}). 1 mL solution each from the last two series of dilutions (10^{-7} and 10^{-8}) were poured into MRSB-agar media and incubated at 37°C for 48 h. Lactic Acid Bacterial colonies were calculated according to Bacteriological Analytical Manual (BAM) 2001 standard based on FAO/WHO (Food and Agriculture Organization/World Health Organization). Calculation of bacterial colonies was carried out with the following formula:

$$N = \Sigma c / \{(1 \times n1) + (0,1 \times n2) \times d\}$$

N = total bacterium

Σc = total number of colonies

n1 = number of plates of the first dilution

n2 = number of plates of the second dilution

d = level of dilution

2.5. Lactic acid levels measurement

Measurement of lactic acid levels was carried out by titration using 0.1 N NaOH. About 5 mL of the fermented samples were taken and phenolphthalein indicators were added, followed by titration with 0.1 N NaOH.

2.6. pH level measurement

Measurement of the pH value was done by taking 10 mL each of the fermented samples, followed by measurement with a pH meter.

2.7. Total reducing sugar level measurement

The measurement of total reducing sugar level was carried out by the Miller method. About 1000 μL of the fermented sample was taken and heated for 1 min. The solution was then centrifuged for 10 min ($4^{\circ}\text{C}/10,000\ \text{rpm}$). 250 μL of the supernatant was taken and 750 μL of Miller reagents were added. The mixture was heated for 15 min (100°C). The mixture was then cooled for 20 min, and its absorbance was measured at 550 nm.

3. Result and discussion

3.1. Hydrolysis of coffee peel xylan by endo- β -1,4-D-xylanase

Hydrolysis of coffee peel xylan by endo- β -1,4-D-xylanase with an activity of 4.53 U/mg produced XOS, identified as xylo-tetraosa ($R_f = 0.40$) and xylopentaosa ($R_f = 0.34$). The identification was done qualitatively by TLC as shown in Fig. 1. XOS from corn hull

Table 1
Variation on MRSB-m bacterial culture media.

Final XOS from Coffee Peel in MRSB-m Concentration (%)	XOS from Coffee Peel Solution (mL)	MRSB-m (mL)	<i>L. casei</i> (mL)	Total Volume (mL)
0	0	9.0	1.0	10.0
10	1.0	8.0	1.0	10.0
20	2.0	7.0	1.0	10.0
30	3.0	6.0	1.0	10.0

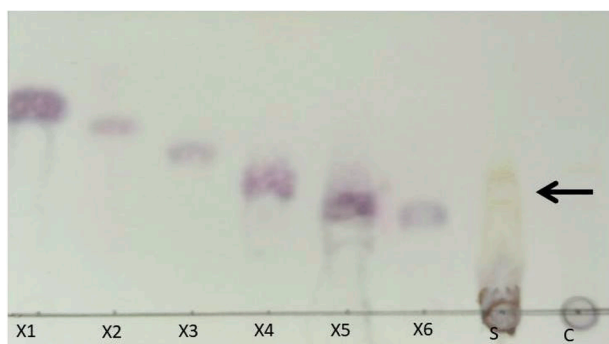


Fig. 1. TLC chromatogram of XOS from coffee peel. X1-X6, standard, S, XOS from coffee peel, C, the negative control (xylan from coffee peel that was not hydrolyzed).

were reported to contain xylobiose, xylotriose, xyloetraose, xylopentaose, xylohexaose and xiloheptaose. This showed that the hydrolysis process randomly broke the β -1,4-glycoside bonds in the main xylan chain, resulting in XOS formation with different degrees of polymerization. Data from the TLC test were confirmed quantitatively with HPLC to produce a chromatogram (Fig. 2.) with a retention time of (RT) 1968 min. Compared with chromatogram data of the standard (X1-X6), it was known that the sample contained xylopentaose (X5) with a concentration of 3.26 mg/mL.

3.2. XOS from coffee peel fermentation

Similar bacterial growth profiles were observed despite additions of varying concentrations of addition of XOS (10%, 20%, 30%) to the bacterial culture media. Bacterial growth increased in the first 12 h of incubation and then gradually decreased throughout the final 48 h of incubation. Increased growth of *L. casei* was also seen in control media (0% XOS coffee peel). These data support the findings of Puspaningsih et al. (2008), who found that the number of *L. casei* on MRSB control media (without XOS) increased for 12 h, and the number was almost constant until the 24 h of incubation. Moura et al. (2007) reported that *Lactobacillus* bacteria growth in corn hulls XOS-supplemented media increased until 30 h of incubation.

L. casei showed exponential growth in the first 12 h of incubation. During this time, cell division occurred rapidly, and mass doubled.

The cells then underwent a stationary phase which showed a proportional rate of cell growth and cell death. Bacterial growth at 12 h was indicated by the total colonies of 7.61, 7.94, 8.75, and 8.26 log CFU/mL for each addition of 0%, 10%, 20%, and 30% XOS from coffee peel in the bacterial growth medium. After that, the total colonies of bacteria gradually decreased until the 48 h of incubation ended (Fig. 3.).

Based on the bacterial growth profile (Fig. 3), addition of 20% XOS from coffee peel resulted in the highest increase in growth (8.75 log CFU/mL) compared to the addition of 0%, 10% and 30% XOS. It could be concluded that the growth of *Lactobacillus casei* could optimally grow in media containing 20% XOS from coffee peel. Based on the study by Moura et al. (2008), XOS from agricultural waste or commercial XOS could increase the growth of *Bifidobacterium* and *Latobacillus* bacteria up to 11.5 h of incubation, at which point the bacteria would go in a stationary phase.

The sugar fermentation process by bacteria would produce lactic acid and short-chain fatty acids. Based on the graph in Fig. 4., total lactic acid titrated from a medium containing 30% of the XOS from coffee peel increased significantly until the 48 h. It was only 0.16% at 12 h, but then it increased rapidly to 0.46% over the next 12 h. After 24 h, there was no significant change. The final fatty acids content measured 0.50% at 48 h. This result was the highest when compared with the result of bacterial growth media supplemented with other concentrations of XOS from the coffee peel. Media with 20% XOS showed greater than in other media that had a low percentage of total lactic acid and did not change much over time (about 0.19%- 0.25% lactic acid). In contrast, the total lactic acid from the media with 0% and 10% XOS showed inconsistent results. This result could be related to the fact that fermentation process did not produce lactic acid as the final product. According to the experiment by Muora et al. (2008), not all XOS from agricultural wastes could produce lactic acid in colon bacteria. The detection of lactic acid in that study was only at 11.5 h, there was no lactic acid detected afterward. Lactate that was formed was actually an intermediate compound that would soon be converted into several other organic compounds such as acetate, propionate and butyrate (Belenguer et al., 2006; Cotto and Whitehead, 1998; Duncan et al., 2004). Study by Puspaningsih et al. (2008) was also showed that the total acetic acid was much greater in media after fermentation.

The level of short-chain fatty acids was analyzed with GC-MS which removes short-chain fatty acids from the fermented media. The

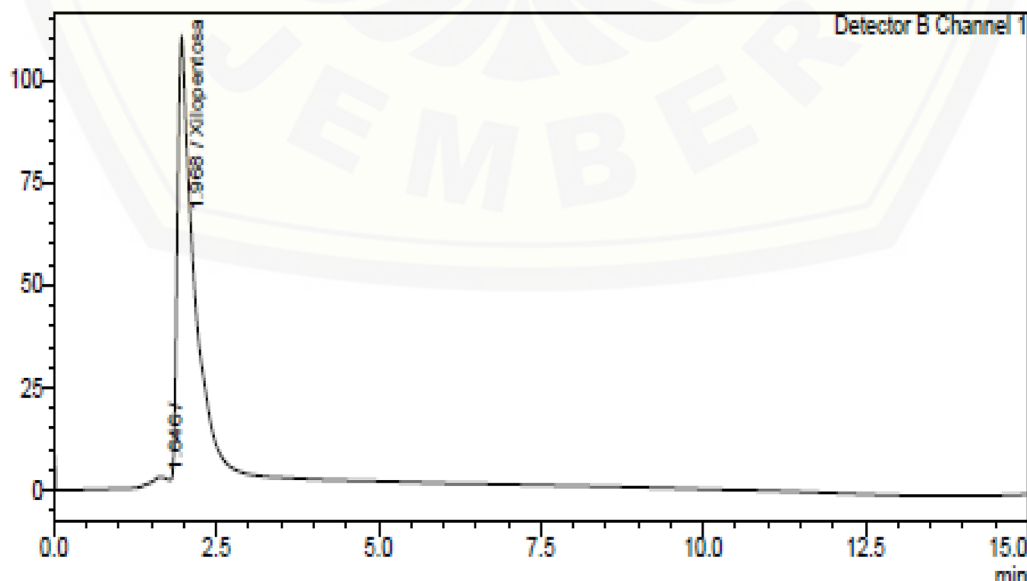


Fig. 2. HPLC chromatogram of XOS from coffee peel.

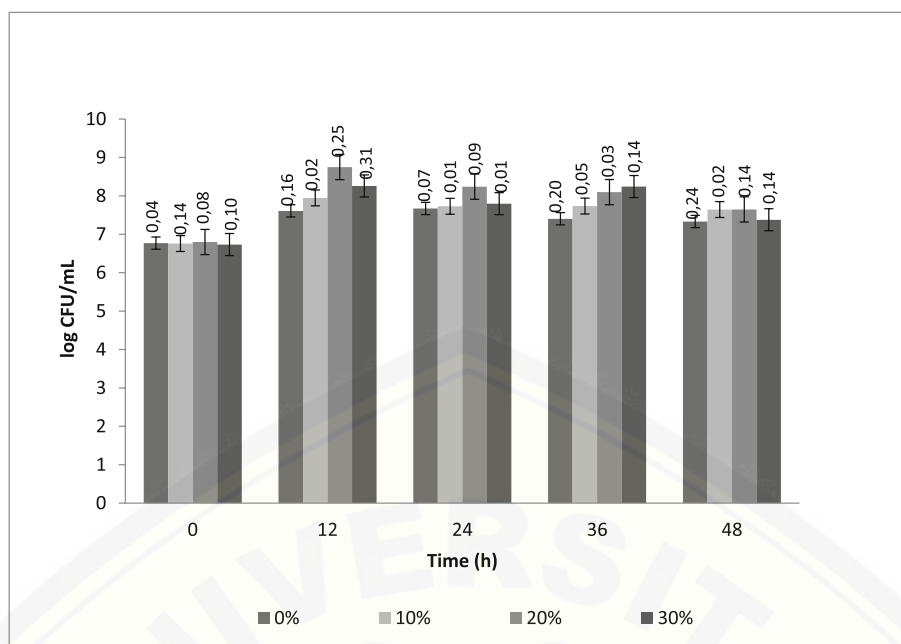


Fig. 3. *Lactobacillus casei* growth on growth medium added with different concentration of XOS from coffee peel. Each value in the panel represents the mean \pm SD.

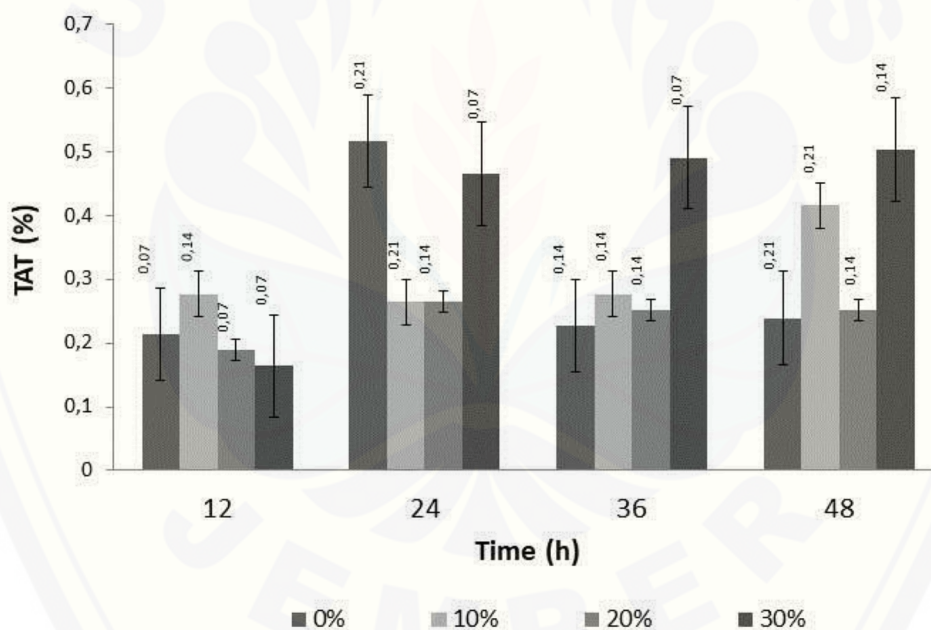


Fig. 4. Total lactic acid titrated (TAT) in fermented media containing 0; 10; 20 and 30% of XOS with each time for 12; 24; 36; and 48 h. Each value in the panel represents the mean \pm SD.

organic compounds that were detected were organic acids with long carbon chains, such as tetradecanoic acid methyl ester, decanoic acid methyl ester, 9-hexadecenoic acid methyl ester, and 11-octadecenoic acid methyl ester. In general, acetate and butyrate were the most abundant organic fatty acids produced via in-vitro fermentation tests (Moura et al., 2008).

This study showed that the pH value of the media decreased during incubation (Fig. 5.). The decrease in pH values of media supplemented with 0% and 20% XOS were from 7.10 (0% XOS) and 7.365 (20% XOS) until 6.00 and 5.84 by 48 h. According to Parra et al. (2015), a similar pattern was also observed in several fermented media for lactic acid bacteria that was supplemented with extract from agro-industrial by-products. Meanwhile, there were irregular changes

in pH values of media supplemented by 10% and 30% XOS. The lowest pH value of 5.49 was found in the media with 30% XOS after 24 h, though the pH value was increased to 5.71 at 36 h. The pH value of 10% XOS in media originally measured 7.40 and then decreased significantly over 12 h to a value of 6.44; 6.29 and 5.85 at 36 h, and then a sudden increase to 5.92. The irregular pH changes are caused by the formation of ammonia (NH_4OH) from NH_3 resulting from the breakdown of amino acids from a medium containing meat and yeast extract. These can increase the pH value.

The reducing sugar level in the media was influenced by the incubation time on the fermentation process by *L. casei*. Based on Fig. 6, reductions in media sugar levels decreased over time. This showed that *L. casei* utilized XOS for cell metabolism. The profile on Fig. 6 in-

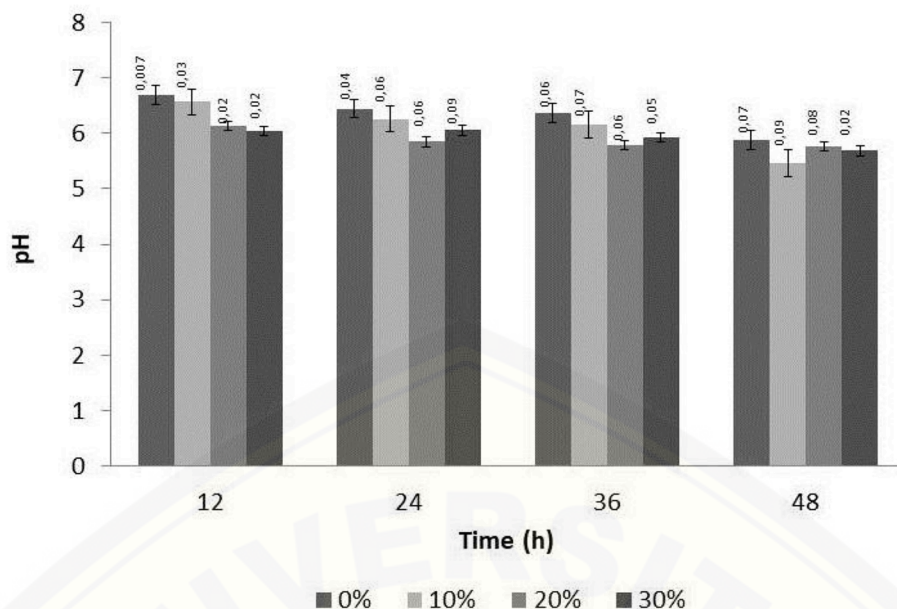


Fig. 5. The profile of pH value of fermented media containing 0; 10; 20 and 30% of XOS with each time for 12; 24; 36; and 48 h. Each value in the panel represents the mean \pm SD.

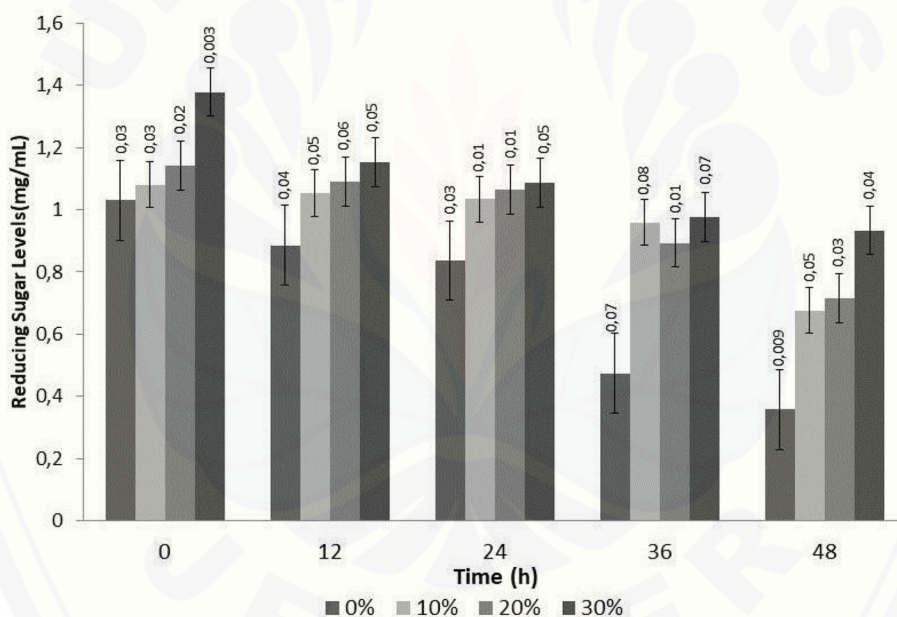


Fig. 6. The profile of reducing sugar levels in fermented media over time. Each value in the panel represents the mean \pm SD.

indicated that the reductions in sugar levels corresponded to amounts of XOS added to the media. Media with 30% XOS showed the greatest reductions in sugar levels (1.38 mg/mL) when compared to other media, i.e. 1.14 mg/mL (20% XOS), 1.08 mg/mL (10% XOS), and 1.03 mg/mL (0% XOS). The presence of reducing sugars at 0% XOS comes from 4 to 10% carbohydrate contained in meat and yeast extracts in the medium. This study showed that XOS added to culture media was readily used as a carbon source by *L. casei* bacteria. This activity caused a reduction in sugar levels. Even after 48 h, after the fermentation process ended, reductions in sugar levels were still fairly high. The reason for this is the continued proliferation of bacteria using the available food sources in the media (Fig. 2).

4. Conclusion

L. casei grew best in media with carbon sources of 20% XOS from coffee peel. Fermentation by lactic acid bacteria produced lactic acid and other organic acids as products that could reduce the pH value of the media.

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