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Research Article

Biotransformation of *n*-butanol to Fruity-Like Bio-Flavour by Indonesian Lactic Acid Bacteria

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ABSTRACT

Microbial production of aroma compounds is a promising alternative to extracting plants or chemical synthesis. In our research, the Indonesian lactic acid bacteria (LAB) have been utilised as producing fruity-like bio flavour by biotransformation approach using n-butanol as a precursor. The aims of our research are to identify LAB- secondary metabolites categorised fruity-like bio flavour and investigate the changes of glucose, mannitol, xylose, lactic acid and acetic acid in growth medium after fermentation. Our result research showed that n-butanol could be transformed to several fruity like bio flavour such as ethyl butyrate, butyl acetate, butyl formate, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 2-heptanone, butyl propanoate, butyl propanoate, butyl 2-methylbutanoate, butyl isovalerate, butyl pentanoate, and butyl hexanoate. All of LABs consumed above 75% of glucose and only *Lactococcus lactis* KGB1 consumed all the mannitol on fermentation medium. In addition, *Lactococcus lactis* KGB1 produced the highest xylose, 11.87 g/L LABs produced. Based on the amount of fruity-like bio flavour compound generated, *Lactobacillus fermentum* WKS2, *Lactobacillus fermentum* KGL2, *Lactococcus lactis* KK4, *Lactobacillus fermentum* WKS3, *Lactococcus lactis* KGB1, and *Lactobacillus fermentum* KGL7 could be considered as agent fruity-like bio flavour by biotransformation approach.

INTRODUCTION

Flavour is an important component of food could improving organoleptic properties by giving satisfaction to consumers. Flavour ingredient is generally added to food in small amounts to impart a specific taste to a product or replace flavours lost during processing. In the food and beverage industry, flavours are needed to create new products, to introduce new products. and to change the taste of existing products. One type of flavour that is in demand in the food and beverage industry

is fruit flavour (Reshna et al. 2022). Worldwide demand of fruity flavor reaches a market valuation to US$ 1.23 billion by 2034 (Fact.MR 2024).

There are three kinds of flavour: synthetic, natural, and bio-flavour. Synthetic flavours can undergo lethal synthesis when introduced into the body's metabolic pathways because of toxic compounds which cause many complex chronic disorders. To reduce this risk, an alternative is the use of natural flavours (bio-flavor) obtained from natural sources such as animals, plants, and microorganisms. Plant-natural flavors have many disadvantages such as being expensive, weak, and not resistant to the rigors of food processing and storage. By biotechnology, bio-flavour could overcome the weakness of plant-natural flavour such as depends on seasonal and climatic, has low concentration, and improves ecological problem (Bicas et al. 2010). Bio-flavour utilises microbes that have many advantages such as resistance to temperature, gas, pH; and unstable gas during food processing. It is also beneficial for improving health (Roy & Kumar 2019).

Bio-flavor compounds can be produced in two ways,namely de novo synthetic pathways and biotransformation processes that involve the addition of precursors, as well as enzymes to help microbes convert a compound to other volatile compounds (Hosoglu et al. 2018). In our research, we used biotransformation methods to produce fruity-like flavour. Biotransformation is a method that has the ability to modify the structure of an organic compound with the help of microorganisms or the addition of enzymes. Some precursors require the presence of enzymes. For example, the precursor butanol and lipase enzyme with the aim of producing more specific bioflavor compound, namely butyl butyrate (Seo et al. 2017). The advantages of the biotransformation method compared to the de novo synthetic method are that it can produce bio-flavor compounds with certainty and it can produce more optimal bioflavor compounds (Shaaban et al. 2016).

LAB (lactic acid bacteria) can produce flavour compounds through biosynthetic pathways such as fermentation. During fermentation, carbohydrates, proteins, and fats are broken down by microbes to produce flavour compounds (Hosoglu et al. 2018). From research conducted by Nor et al. (2021). LAB of the genus *Lactobacillus* sp. is known to be able to produce bioflavor compounds of ester groups and their derivatives such as methyl esters with fragrance characteristics of fruits and flowers. *Lactiplantibacillus plantarum* is one of the LAB group bacteria known to be able to produce ester-derived compounds naturally in the form of butyric acid (Aiello et al. 2023). In our study, we employed LAB on producing fruity-like bio-flavour. The aims of this research are to analysed the volatile organic compounds (VOC) generated and the changes of sugar composition (glucose, mannitol, xylose) also organic acids (lactic acid and acetic acid) in fermentation medium. To triggers LAB in producing bioflavor compounds, *n*-butanol and lipase were added as a precursor and a as a catalyst for the esterification process.

MATERIALS AND METHODS

Materials

This research screened 18 LABs isolate, namely *Lactococcus lactis* KGP1, *Lactobacillus fermentum* KBP2, *Lactobacillus fermentum* IPEA, *Lactococcus lactis* KK4, *Lactobacillus fermentum* WKS3, *Lactococcus lactis* KGP2, *Lactococcus lactis* KGP3, *Lactobacillus fermentum* KGL7, *Lactococcus garvieae* SS3, *Lactobacillus kefiranofaciens* KK2, *Lactococcus garvieae* SS5, *Lactococcus lactis* KGB1, *Leuconostoc mesentroides* KGL2, *Lactococcus garvieae* SS4, *Lactobacillus fermentum* KGG3, *Lactobacillus kefiranofaciens* KK1, *Lactococcus lactis*

KGB3, *Lactobacillus fermentum* WKS2. All those isolates were private collection of our group research. The reagents used in this research were de Man Rogosa Broth (Merck, Germany). Bio-flavour synthesis- fermentation medium containing lipase (technical grade, as catalysing enzyme). *n*-butanol (Merck, Germany. as precursor), yeast extract (Himedia, India), glucose (Himidea, India), 0.5 g/L KH_2PO_4;0.5 g/L K_2HPO_4; 2.2 g/L CH_3COONH_4; 0.2 g/L $MgSO_4.7H_2O$; 0.01 g/L $MnSO_4.H_2O$; 0.01 g/L $FeSO_4.7H_2O$; 0.01 g/L NaCl; 10 µg/L, biotin. All of those minerals are produced by Merck, Germany. Yeast and glucose solution were sterilised by autoclave at 121°C, 15 minutes and mineral solution was filtered by 0.45 µm membrane.

Methods

Fermentation process

The glycerol stock of LABs was cultured in de Man Rogosa Broth (MRSB) for 24 hours, 30°C, twice. The 10^8 colony forming unit (cfu)/ml - working culture was inoculated into medium fermentation and incubated at 30°C for 48 hours. After fermentation, all samples were harvested and centrifuged at 4°C, 10000 rpm, 10 minute. The supernatant was collected and stored at -20°C for further analysis.

Analysis of fermentation products

VOCs were obtained using headspace Gas Chromatography Mass Spectra (headspace-GC-MS) Shidmadzu QP 2020. 2 ml of the supernatants were placed in headspace vial and injected into GC-MS through headspace methods. The samples were equilibrated at 60°C for 20 minutes. 2000 µL of the sample's vapor was injected into GC-MS. The initial GC temperature was set at 40°C for 1 min with a ramp rate of 5 C/min to 70°C, increased at the rate of 10 C/min to 220°C, held for 1min. Samples were introduced into the split ratio 1:10 at 230°C at a pressure of 61.8 kPa with helium carrier gas. with a purge flow 2.3 mL/min. A RTx-5Sil-MS column (30-m length 0.25-mm i.d. 0.25-µm df) (Shimadzu) was used for all analyses. Purge time was set at 1 min. The MS transfer line was maintained at 250°C and ion source at 230°C. All samples were analysed using scan mode from 50 to 550 m/z.

Glucose, mannitol, lactic acid and acetic acid in the aqueous medium were quantified by a high-performance liquid chromatography (HPLC) (Shimadzu) system equipped with a refractive index detector (RID) using ICSep COREGEL-87H column (300 x 7.8 mm). The column was eluted with 5mM of H_2SO_4 at a flow rate of 0.6 mL/min at 30°C.

Statistical analysis

All results were expressed as a mean of two replicates. The changes in glucose, mannitol, lactic acid and acetic acid were analysed by descriptive analysis. Principle Component Analysis (PCA) was employed to investigate the sample groupings and correlations among volatile profiles of all isolates. The data was carried out using XLStat (Version 2019 v.2.2), and an add-in software package for Microsoft Excel (Addinsoft Corp., Paris, France).

RESULTS AND DISCUSSION

Principle Component Analysis (PCA) describes the relative location of volatile compounds in bio-flavour metabolites of LABs. The PCA biplot explained about 53.4% of the variability. Most of the variability 38.8%. was attributed to PC1, with PC2 (the vertical axis) accounting for just 14.6% of the total variability (Figure 1). Bio-flavour metabolites of KK1

and WKS2 were quite different compared with other isolates. The highest factor loading metabolite of PC1 was butyl methyl propanoate processed by WKS2. Meanwhile, ethyl 3-methyl butanoate was the highest in PC 2 generated by WKS3. These flavours have apple, pineapple and sour aroma. Figure 1 also explained that biosynthesis of ethyl 2-methyl butanoate had a negative correlation with butyl methanoate. It meant that the number of ethyl 2-methyl butanoate's biosynthesis was opposite with butyl methanoate. Butyl butyrate and butyl acetate as the main VCOs were present in KK4 and WKS2.

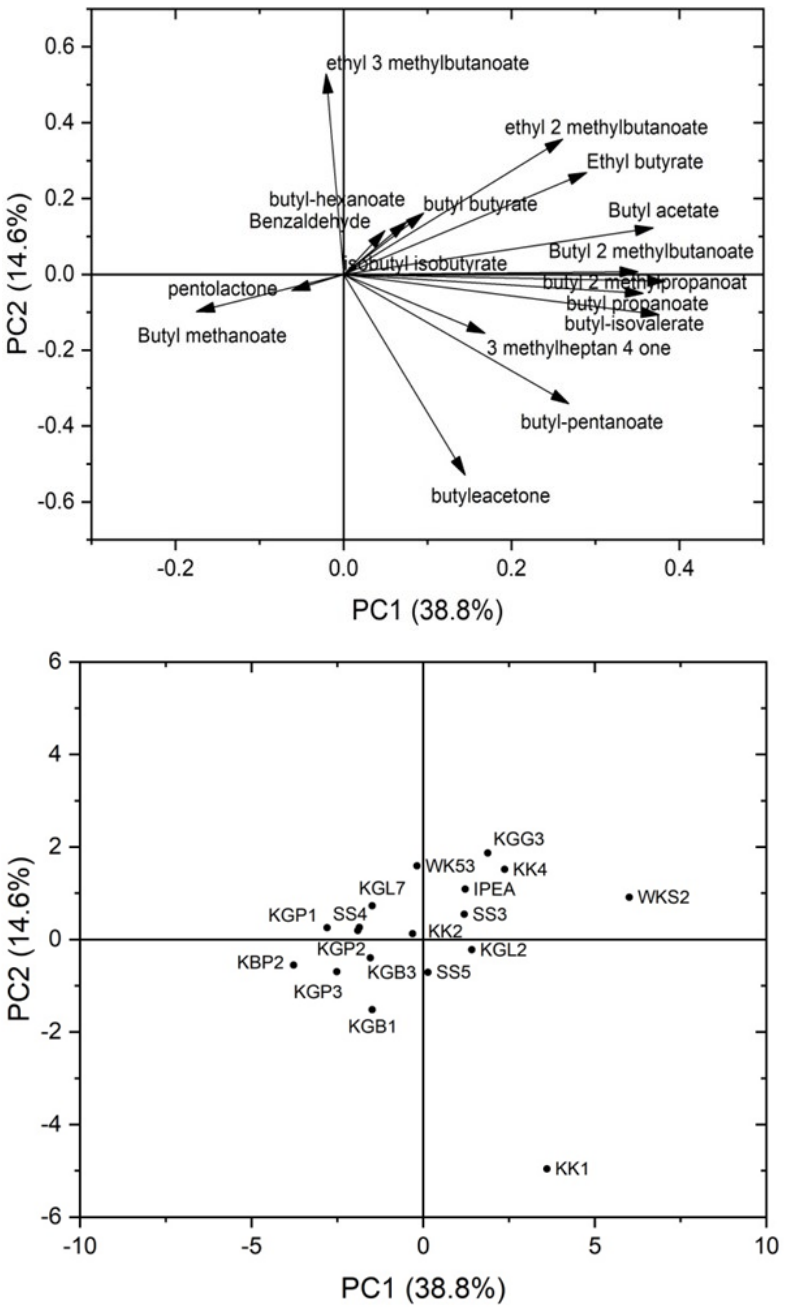

Figure 1. Principle component analysis biplot VCOs detected in LABs through *n*-butanol biotransformation.

Lactic Acid bacteria (LAB) naturally have been known to have the ability to synthesise some organic flavours such as ester and ketone that have been used in food production. Several studies showed that LAB can produce certain flavor compounds such as butyric acid (Gupta 2015), vanillin (Kaur et al. 2013), and diacetyl (Leroy & De Vuyst 2004). The bio-flavour produced by LABs was shown in Table 1. Among 17 compounds analysed, butyl acetate (BA) and butyl butyrate (BB) formation

were mostly produced among the isolates. The pathway was through alcohol acyltransferase (AAT). Both two compounds were derived from butanol; a precursor that was supplemented in the media. Butyryl-CoA was metabolically synthesised from glucose and mannitol (building block) and conducted a condensation reaction with butanol to form BB by AAT (Guo et al. 2023). The addition of other sugar like mannitol was to increase the amount of fructose-6-P in order to produce more pyruvate.

Analogous to BB, the synthesis of BA was also through AAT (Figure 2). Instead of butyryl-CoA, the acetyl-CoA was synthesised to form BA. Compared to butyryl-CoA synthesis, acetyl-CoA formation was simpler since it was less carbon and formed after the pyruvate step (Ku et al. 2022). In the same isolate, the amount of BA tended to be higher than BB. That might be caused by the pathway to form BA was shorter than the BB.

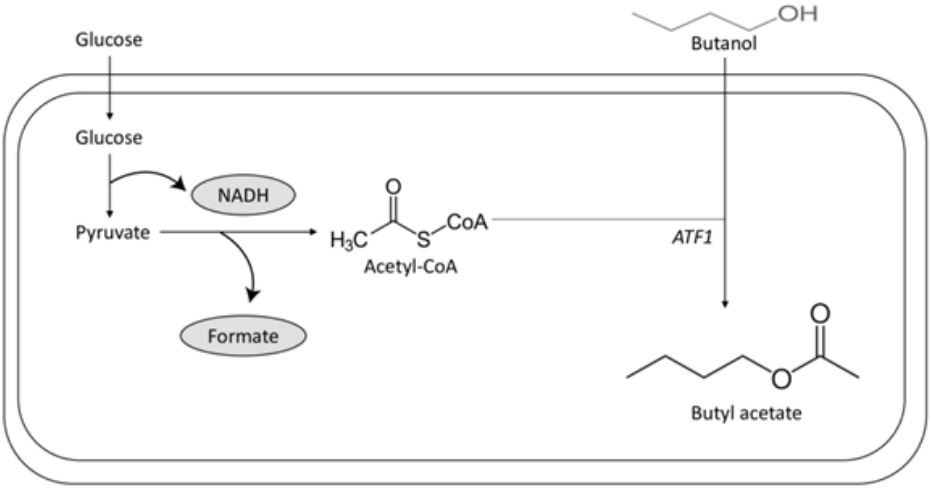

Figure 2. Butyl acetate pathway from glucose with butanol supplementation.

The three bacteria that produced the highest BA were *Lactobacillus fermentum* WKS2, KGL2, and *Lactococcus lactis* KK4 produce the highest BA with 79.30, 73.69 and 71.70% from the total bio-flavour produced respectively. On the other hand, the other bacteria that produced the highest BB were *Lactobacillus fermentum lactis* KGB1 and *Lactobacillus fermentum* KGL7 with 54.17, 52.17 and 50.41% from the total bio-flavour produced respectively (Table 1) Based on the sugar consumption,almost of isolate consumed 100% glucose in the fermentation media (compare against the media without fermentation) except for *Leuconostoc mesenteroides* KGL2 which only consume 75% of the glucose presence (Figure 3). These means that glucose was a carbon source that used to synthesise metabolites and energy carrier. Even though glucose in media had been consumed by all of isolates, the differences of concentration BB formation probably due to the differences in AAT activity in each isolate. ATT plays a role in condensation between butyryl-CoA and *n*-butanol (Noh et al. 2019).

In terms on mannitol consumption,only *Lactococcus lactis* KGB1 that consumed all the mannitol presence in the fermentation media, the second and the third highest were KGP2 and WKS3 with 60% mannitol consumption (Figure 3). Though many exception, mannitol is commonly fermented by LABs and produce lactate following path : mannitol à mannitol-1- P à fructose-6-P à 2 pyruvate à2 lactate (Liu 2003). Not many LABs have been reported be able to metabolise mannitol. *Lactobacillus plantarum* and *Lactobacillus casei* were reported can ferment mannitol to lactate and other metabolites, depends on the presences of oxygen (Liu

2003). The relatively high BB produced by *Lactococcus lactis* KGB1 might because it maximised the sugar consumption so that the amount of Acetyl Co-A was higher than other bacteria. Mannitol consumption was less than glucose because glucose prevents the use of other carbon sources. Furthermore,many bacteria prefer glucose as their carbon source including LAB. Mannitol was more reduced than glucose, thus it can form more NADHs (3 mol) than glucose (2 mol) (Fu et al. 2020). Glucose fermentation was not only synthesising the flavouring compound, but also producing another compound such as lactic acid, acetic acid, and xylose. These compounds were formed by different metabolism. Lactic acid, acetic acid, and xylose are produced via hetero-lactic metabolism (PK-pathway) and homo-lactic acid metabolism (PP-glycolytic pathway) (Abedi & Hashemi 2020).

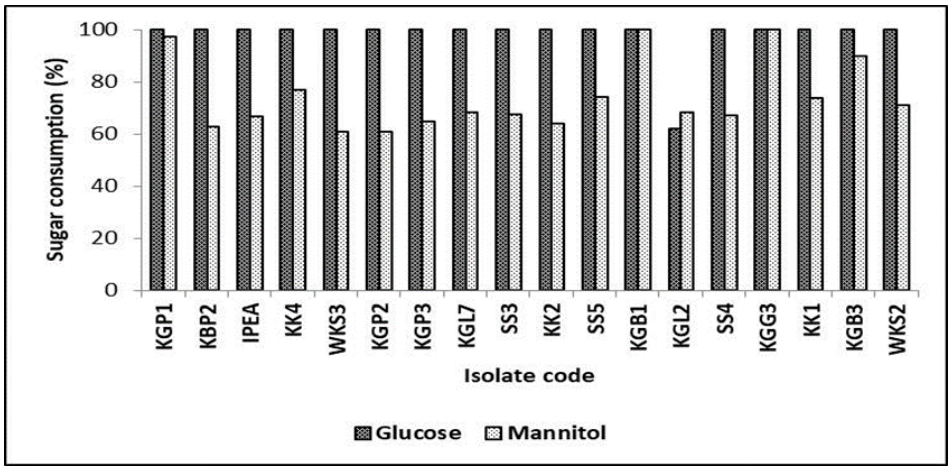

Figure 3. Glucose and mannitol consumption of LABs

Over 90% of lactic acid was produced through microbial fermentation (Rodrigues et al. 2015). Lactic acid can be produced both via PK-pathway and PP-glycolytic pathway. In both paths, the glucose was converted into glucose 6-P. Glucose 6-P was then converted into 6-Phospho-Gluconate in PK-pathway, while in PP-glycolytic pathway, it was converted into fructose 6-P. In the end, both of the substituent were converted into pyruvate and then they were formed lactic acid by oxidising the NADH into NAD^+.

Lactobacillus kefiranofaciens KK1, *Lactobacillus fermentum* WKS2, and *Lactobacillus fermentum* KGL2 produced the highest lactic acid which were 16.34, 13.37. and 12.88 g/L respectively (Figure 4). Abedi et al. (2020) has reviewed some LAB producing lactic acid. It showed that *Lactococcus lactis* with glucose as a carbon source can produce lactic acid between 0.3 g/L to 39 g/L. In addition, *Lactobacillus fermentum* produced lactic acid between 5.19 to 31.11 g/L.

Acetic acid was only produced via PK-pathway. Acetic acid was produced after acetyl-P was formed. It can be from xylulose 5-P or from acetyl-CoA by phosphate transferase. Acetic acid was considered as a side product in lactic acid production. To some extent it was undesired product and also potentially inhibit the bacteria environment since the pH level would getting lower.

In the end of the fermentation, xylose was also produced as an intermediate sugar. It was begun to form when xylulose-5-P was formed. Xylose was equilibrium with xylulose-5-P and xylulose. Xylulose was formed by removing the phosphate group and bonding with ADP to form ATP. After that, the xylose was formed by the assist of xylose reductase and xylitol dehydrogenase (Abedi & Hashemi 2020). Figure 5 showed

Table 1. Fruity bio-flavour produced by LABs.

RT	Compound	Flavour description	Isolate code[A]																
			KGP 1	KBP 2	IPEA	KK4	WKS 3	KGP 2	KGP 3	KGL 7	SS3	KK2	SS5	KGB 1	KGL 2	SS4	KGG 3	KK1	KGB 3	WKS 2
			% area[BC]																
4.629	Ethyl butyrate	Apple. Pineapple. strawberry	nd	nd	3.10	2.57	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	2.92	nd	nd	3.41
4.900	Butyl acetate	Apple. banana	58.13	nd	69.99	71.7	27.79	61.31	nd	nd	70.28	63.41	64.77	nd	73.69	nd	68.86	63.38	46.35	79.3
4.913	Butyl formate	Fruity	nd	44.32	nd	Nd	nd	nd	42.39	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
5.730	Ethyl 2-methylbutanoate	Apple. strawberry	nd	nd	0.22	0.29	0.79	nd	nd	1.85	0.33	0.7	0.65	nd	0.31	2.07	0.64	nd	nd	0.52
5.835	Ethyl 3-methylbutanoate	Apple. pineapple. sour	2.22	1.56	0.66	0.84	1.14	1.71	1.67	3.32	0.76	0.57	0.69	nd	0.29	3.54	0.89	nd	1.58	0.24
6.760	Hepta*n*-2-one	Nutty. Spices	nd	nd	nd	Nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.18	nd	nd
7.240	Butyl propanoate	Strawberry	9.9	8.26	4.57	4.23	5.84	5.83	8.63	16.53	5.03	5.96	5.54	17.67	4.68	14.31	4.71	6.16	7.96	2.68
7.692	Butyl propyl ketone		nd	nd	0.19	0.16	nd	0.4	0.61	nd	0.17	nd	0.24	1.04	0.13	0.86	0.11	0.19	0.49	0.11
8.350	Butyl isobutyrate	Fruity	11.95	11.52	5.84	5.19	7.16	7.7	10.66	19.67	6.11	7.87	6.91	19.51	4.94	19.01	5.40	7.31	10.32	3.72
8.632	Benzaldehyde	Almond	nd	nd	0.12	Nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
9.333	Butyl butyrate	Apple. banana. pineapple	14.5	29.53	13.02	12.86	54.17	19.81	31.44	50.41	14.86	17.59	17.11	52.17	13.63	50.25	13.93	18.97	28.3	7.72
9.717	DL-pentolactone		nd	nd	nd	Nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.16	nd
10.30	Butyl 2-methylbutanoate	Fruity	nd	1.65	0.77	0.71	1.12	1.18	1.63	2.82	0.9	1.21	1.13	2.84	0.74	3.18	0.79	0.99	1.57	0.61
10.41	Butyl isovalerate	Fruity	3.3	3.17	1.52	1.39	1.99	2.05	2.97	5.4	1.55	2.68	2.81	6.76	1.58	6.78	1.75	2.64	3.27	1.71
11.32	Butyl pentanoate	Fruity	nd	nd	nd	0.07	nd	nd	nd	nd	nd	nd	0.15	nd	nd	nd	nd	0.17	nd	0.05
12.44	Butyl hexanoate	Fruity	nd	nd	nd	0.07	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

[A]Isolate code: *Lactococcus lactis* KGP1, *Lactobacillus fermentum* KBP2, *Lactobacillus fermentum* IPEA, *Lactococcus lactis* KK4, *Lactobacillus fermentum* WKS3, *Lactococcus lactis* KGP2, *Lactococcus lactis* KGP3, *Lactobacillus fermentum* KGL7, *Lactococcus garvieae* SS3, *Lactobacillus kefiranofaciens* KK2, *Lactococcus garvieae* SS5, *Lactococcus lactis* KGB1, *Leuconostoc mesentroides* KGL2, *Lactococcus garvieae* SS4, *Lactobacillus fermentum* KGG3, *Lactobacillus kefiranofaciens* KK1, *Lactococcus lactis* KGB3, *Lactobacillus fermentum* WKS2

[B]nd = not detected

[C]mean of two replicates

that all bacteria produced xylose less than 2 g/L except for *Lactococcus lactis* KGB1 which surprisingly produced 11.87 g/L of xylose. Xylose has fewer calories than table sugar so that it is used as a diabetic sweetener in food and beverage (Galvan et al. 2022).

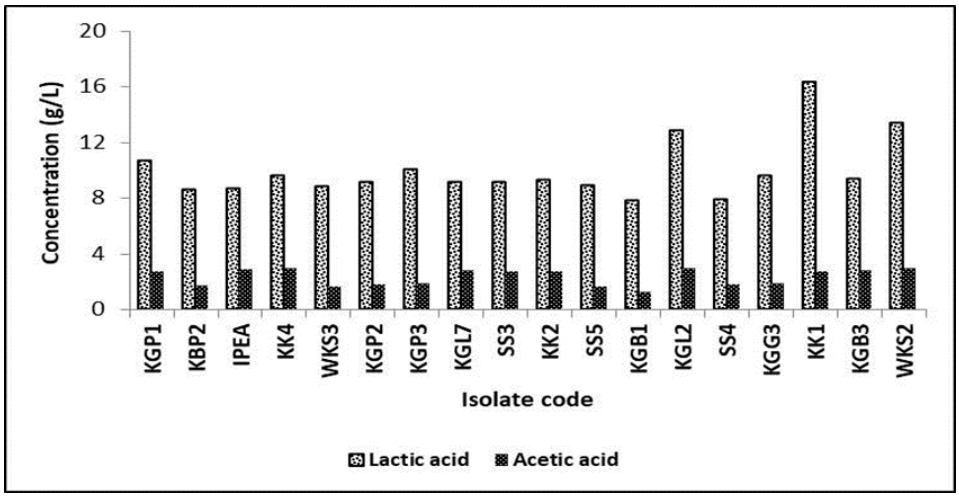

Figure 4. Lactic acid and acetic acid production of LABs.

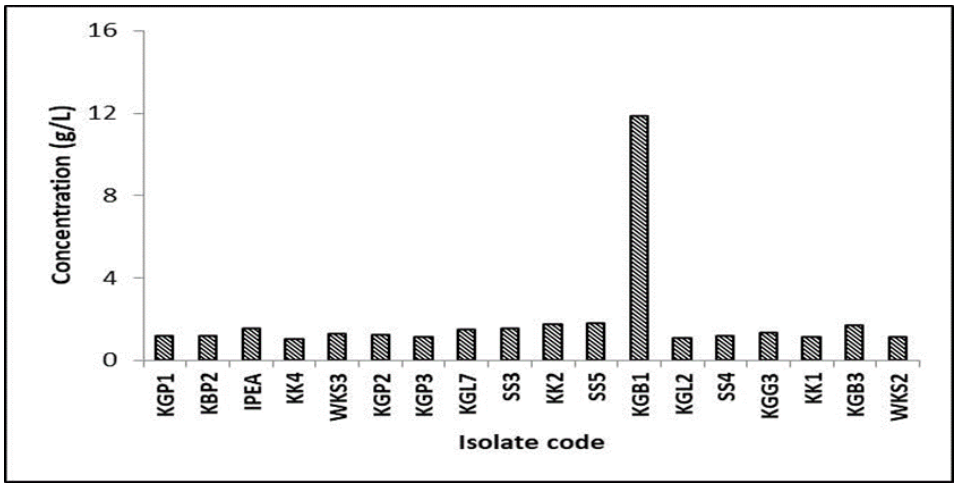

Figure 5. Xylose production from LAB.

CONCLUSIONS

All employed LABs generated fruity-like bio-flavour such as butyl acetate (BA), butyl butyrate (BB), ethyl 3-methylbutanoate, butyl propanoate, butyl 2-methylpropanoat and butyl isovalerate. The most VOCs resulted through *n*-butanol transformation were butyl acetate and butyl butyrate which have apple, banana, and pineapple aroma. The highest BA was produced by *Lactobacillus fermentum* WKS2, meanwhile the highest BB was *Lactobacillus fermentum* WKS3. Almost all LABs consumed 100% glucose in the fermentation media except *Leuconostoc mesenteroides* KGL2. There was only *Lactococcus lactis* KGB1 which consumed all mannitol presence in the media and produced above 10 g/L of xylose in media fermentation. In terms of organic acid, *Lactobacillus kefiranofaciens* KK1 produced the highest lactic acid and acetic among others.

AUTHOR CONTRIBUTION

F.S designed the research and supervised all the process; DTNA, GP, DSW, DDP, FA contributed on collecting data; SO, RF, ARS, DV analysed the data. All authors contributed on writing the manuscript.

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CONFLICT OF INTEREST

There is not any conflict of interest regarding the research or the research funding.

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