

The Potential of Clove Rhizospheric Bacteria to Produce Vanillin from Eugenol

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ABSTRACT

Vanillin is one of the most important flavoring agents worldwide. Currently, consumers' awareness and concern for biovanillin production has been increasing. This study aimed to screen the potential of clove rhizospheric bacteria isolates producing vanillin through a biotransformation process of eugenol and to conduct the preliminary optimization of the biotransformation condition. Twenty-eight bacteria isolates were screened for their capability to transform eugenol into vanillin. BKL 15 isolate, which was identified as *Lysinibacillus xylanilyticus*, was selected as the highest vanillin producer among the isolates. The optimum molar yield of vanillin produced by the selected isolate was 4.99% (1.11 g/L) after 168 hours of biotransformation process in the medium consisting of TSB (30 g/L), eugenol (24 g/L), yeast extract (20 g/L), and concentration of casamino acid (20 g/L). Throughout the publications we have read, this is the first report of *L. xylanolyticus* that produces vanillin.

1. Introduction

The market-scale demand for safe and healthy food additives has increased. This was also followed by the development of various new compounds in the cosmetic and pharmaceutical fields, thus becoming a big opportunity in the bioindustry sector to produce compounds using biotechnology. Various aromatic compounds have an important role, especially in the food and chemical industries. One of the compounds that has been known to play a role in this field is vanillin (Singh *et al.* 2018). Vanillin is considered as one of the most important flavoring agents worldwide. Natural vanillin is obtained through the extraction process of pods of Vanilla plants (Kumar and Balamohan 2013).

Based on data from FAOSTAT (2021), the production of natural vanillin in Indonesia in 2015-2019 was recorded as the second highest in the world (2298 tons) after Madagascar (3077 tons). However, the production costs unit is very high, and there is still a large gap between production capacity

and global market demand for the natural flavor. Therefore, a safe, low-cost, and environmentally friendly alternative technology to produce vanillin is needed. Microorganisms could produce vanillin through a biotransformation process from several cheap and abundant available materials or compounds such as eugenol, guaiacol, and lignin (Mulyono 2012).

Various kinds of microorganisms have been reported for their ability to transform eugenol (Mishra *et al.* 2013). Such bacteria, i.e., *Pseudomonas fluorescens*, *Pseudomonas putida*, *Corynebacterium* sp., *Bacillus safensis*, *Bacillus subtilis*, *Geobacillus* sp. and fungi i.e. *Fusarium solani*, *Byssoschlamys fulva*, and *Penicillium simplicissimum*, have been reported for their ability to transform eugenol (Tadasa and Kayahara 1983; Furukawa and Nagasawa 1998; Priefert *et al.* 2001; Giedraityte and Kalediene 2014; Chen *et al.* 2016; Singh *et al.* 2018). The application of biovanillin produced from the biotransformation process is very broad and has important functions from an economic point of view (Ashengroph *et al.* 2011).

Indonesia is one of the largest clove oil-producing countries in the world. In 2011, the number of

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Indonesian clove oil exports to foreign countries reached 75% or around 4,500–6,000 tons. Clove (*Syzygium aromaticum*) is a native spice plant of Indonesia that is currently also cultivated in several countries around the world. Eugenol is the main compound contained in cloves, each consisting of 90–95% flowers, 83–95% flower stalks, and 82–87% leaves (Tursiloadi *et al.* 2015). Due to its non-mutagenic and non-carcinogenic related properties, eugenol is designated as one of the compounds that has been considered safe by the Food and Agriculture Organization of the United Nations (Raja *et al.* 2015). In addition, eugenol belongs to a group of compounds that are easily obtained and structurally modified to produce their derivative compounds (da Silva *et al.* 2018).

In this study, we isolated and screened the clove rhizospheric bacteria isolates producing vanillin from eugenol and conducted the preliminary optimization of the selected isolate to produce the flavor compound.

2. Materials and Methods

2.1. Chemicals

Standard compounds used in this research, such as eugenol (99%), vanillin (99%), ferulic acid (99%), and vanillic acid (98%), were obtained from Sisco Research Laboratories Pvt (Maharashtra, India). Solvents for high-performance liquid chromatography (HPLC), such as methanol, acetic acid, and other chemicals used, such as ethyl acetate and methanol, as well as those involved in medium components, were obtained from PT. Merck Chemicals and Life Sciences (MCLS).

2.2. Isolation of Eugenol Transforming Bacteria

The bacteria were isolated from the soil samples of the clove plant *Syzygium aromaticum* by the enrichment method. Soil samples were collected from a clove *Syzygium aromaticum* plantation (Bengkulu, Indonesia). Soil sampling was carried out at a depth of ± 10 cm. A total of 2.5 g of soil sample was put into a bottle that contained 5 ml of sterile distilled water. These suspensions were then inoculated into tripton soy broth (TSB) medium (ingredients gram per liter of distilled water): tryptone, 17.0; soytone, 3.0; glucose (dextrose), 2.5; sodium chloride, 5.0; dipotassium phosphate, 2.5; pH 7.3 \pm 0.2 supplemented with 24 g/L eugenol as sole carbon and energy source.

The samples were then incubated aerobically for 48 hours at 28°C with agitation at 150 rpm. One hundred microliter sample cultures were streaked on tripton soy agar (TSA) medium. Morphologically different bacterial isolates on the isolation medium were purified on a TSA medium containing eugenol. Isolates that were purified and capable of growing on a medium containing eugenol were stored in a glycerol medium at four °C for future use.

2.3. Screening of the Isolates

The isolates were cultured in the sterile TSB medium containing 24 g/L eugenol for 48 h in the shaker incubator at 150 rpm. A control experiment was carried out in the same way but without inoculation. After incubation, the cultures were centrifuged for 10 minutes at 10,000 g. The supernatant was acidified with HCL until pH two and extracted using ethyl acetate solvent 1:1 (v/v). The organic phase was separated and evaporated using a rotary evaporator at 50°C. The crude extracts were resuspended in 2 ml of aqueous methanol (50% v/v) before HPLC analysis to determine their vanillin content (Singh *et al.* 2018).

2.4. Characterization of the Potential Isolates

Two isolates with the highest vanillin production were selected and further characterized morphologically and molecularly. Morphological characterization, including observations on shape, color, elevation, margin, and colony size. Genomic DNA isolation was carried out using the quick-DNATM soil microbe miniprep kit following the established procedure. DNA purity and concentration were measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific 2009). Amplification of the 16S rRNA gene was carried out using Polymerase Chain Reaction (PCR) with targeted fragment $\pm 1,300$ bp using 63f and 1387r primers (Marchesi *et al.* 1998). PCR was carried out for 30 cycles; the pre-denaturation stage was at 94°C for 5 min, denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec, extension at 72°C for 1 min, and final extension at 72°C for 10 min. The PCR products were electroporated (at 90 Volts for 30 min) on 1% agarose gel and stained with gel red (Thomas Scientific) before visualizing. The PCR product was sequenced in First Base (Selangor, Malaysia). The resulting sequences were aligned using the Clustal W program and compared with the data in GenBank using the BLASTN program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Phylogenetic and molecular evolutionary analysis was performed using Molecular Evolutionary Genetics Analysis (MEGA) software version 10 (<https://www.megasoftware.net/>) using the Neighbor Joining method and bootstrap analysis with 1,000 replications.

2.5. Determination of Vanillin by HPLC

The extracted sample were filtered using a 0.22 μm PTFE syringe filter. HPLC analysis was conducted using phenomenex C18 column, column temperature at 30°C, mobile double-distilled water (ddH₂O): glacial acetic acid (99:1) as eluent A and 99% methanol as eluent B, 10 μL injected volume, ratio gradient elution 60:40 in 22 min, flow rate of 1 ml/minute. Vanillin in the samples was detected at 270 nm. Each experiment was run four times, and the data shown are the means of four separate experiments with the standard deviation (Ashengroph *et al.* 2011).

2.6. Preliminary Optimization of The Culture Conditions

Optimization of medium composition was conducted by observing the augmentation effect of eugenol, yeast extract (YE), and casamino acids (CA) in various concentrations to biovanillin production. The selected isolate was cultured for 168 hours in 100 ml TSB containing 12, 18, 24, and 30 g/L eugenol to determine the optimum substrate concentration. Both YE and CA concentrations were optimized on TSB, which contained the optimum substrate concentration. A series of concentrations (5, 10, 15, 20 g/L) of both YE and CA were tried. Further

optimization was performed to determine the optimum incubation time, which was conducted in one to six days. The eugenol transformation process on an uninoculated medium (control) was carried out under the same conditions (data not shown).

2.7. Identification of Biotransformation Compounds

Identification of compounds in the crude extract of selected isolates was carried out by GC-MS analysis at the Integrated Laboratory of Bioproduct, National Research and Innovation Agency. The analysis was conducted using a GC-MS instrument equipped with SH-Rxi-5Sil MC Cap. column (30 m \times 0.25 mm \times 0.25 μm). The temperature of the column oven was 50°C injection temperature was 275°C column pressure was 112.0 kPa, and column flow was 1.90 ml/min, the flow rate of the sample was 8.7 ml/min, the linear velocity of the sample was 50 cm/sec, and flow of the sample purge was 3 ml/min.

3. Results

3.1. Eugenol Transforming Bacterial Isolates

Twenty-eight bacterial isolates were obtained from the clove rhizosphere, which was able to grow on a medium containing eugenol. There were five bacterial isolates, namely BKL 1, BKL 3, BKL 9, BKL 15, BKL 16, and BKL 21, which were selected for further optimization because they produced relatively higher vanillin concentrations compared to other isolates (Figure 1). However, isolate BKL 15 was later selected for further characterization because it was the most stable isolate for growth in the

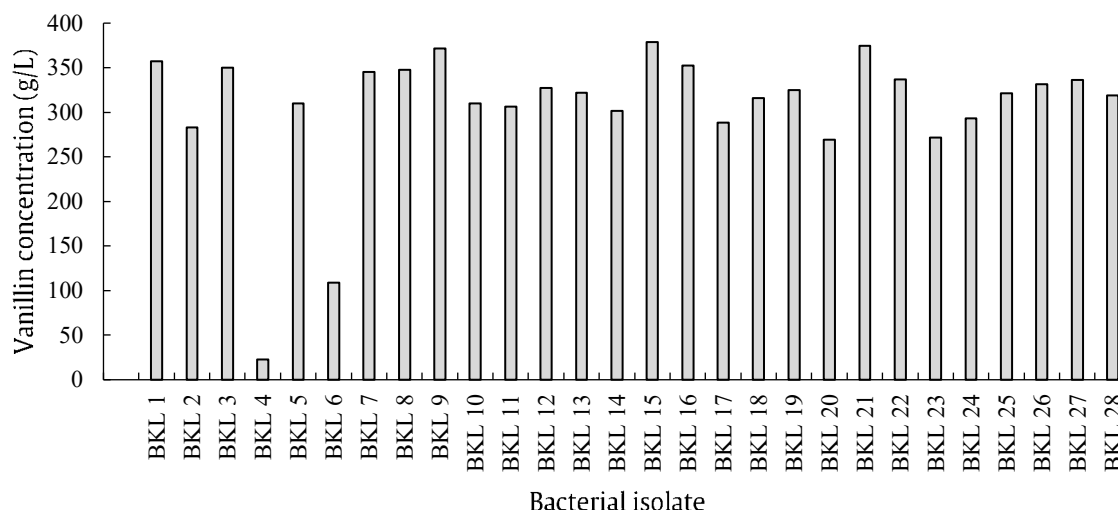


Figure 1. The efficiency of vanillin concentration 28 bacterial isolates

medium and vanillin production. Authentic standard retention times obtained in the method used were as follows: eugenol ± 13.7 min, ferulic acid ± 7.0 min, vanillin ± 6.2 min, and vanillic acid ± 5.4 min (Figure 2). Several derivatives of methoxyphenol compounds produced during the biotransformation process were also identified by comparing them with external standards.

3.2. Characterization of BKL 15 Isolate

BKL 15 bacterial colonies were smooth in shape, had a pinpoint surface, had a flat elevation, had an entire edge, and had a pale-yellow colony color (data not shown). The Gram staining result showed that BKL 15 included Gram-positive bacteria with rod shape. Molecular analysis showed that the BKL 15 had a 98% similarity with the bacterium *Lysinibacillus xylanilyticus* strain XDB9 (accession of GenBank no. NR116698.1) with a strong bootstrap clade value (97/100) (Figure 3).

3.3. Preliminary Optimization of The Culture Conditions

Our findings showed that there were several derivative compounds, such as ferulic acid, vanillin, and vanillic acid, which were detected in the supernatant culture after 168 hours of the biotransformation process. Except for vanillin, the derived compounds were present in small concentrations. The results of the analysis on the effect of different eugenol concentrations showed that the highest vanillin production was achieved under the addition of 30 g/L of eugenol (Figure 4). At this concentration, the result of vanillin production can reach 0.61 g/L (molar yield of 2.23%). For observing the effect of yeast extract and casamino acid, the optimum vanillin production reached 1.11 g/L (4.99% molar yield). These results were obtained after the addition of two mediums to the culture of 20 g/L (Figure 5). Vanillin production based on the difference in incubation time showed that the optimum vanillin concentration occurred on the fourth and fifth days of 0.37 g/L (molar yield 1.66%), and the accumulation tended to decrease on the sixth day of incubation (Figure 6).

3.4. Identification of Biotransformation Compounds

The GC-MS chromatogram of the crude extract of BKL 15 isolate yielded 20 peaks (Figure 7). Table

1 shows the five compounds with the highest concentrations, namely ethylbenzene (retention time ± 5.25 minutes), phenol 4-(2-propenyl)- (retention time ± 15.19 minutes), eugenol (retention time ± 17.35 minutes), vanillin (retention time ± 17.91 minutes), and bis(2-ethylhexyl) phthalate (retention time ± 42.65 minutes). The chemical structure of the five main compounds in the BKL 15 isolate crude extract is shown in Figure 8. The results of mass spectroscopic analysis showed that all detected compounds had a peak area percentage of 0.18-85.96%.

4. Discussion

The 28 clove rhizospheric bacteria isolated from Bengkulu were able to grow on the medium containing 2.4% (w/v) eugenol. All isolates were also detected to have the capability to produce vanillin (Figure 1). The first report on microorganisms that could use eugenol as a carbon and energy source was published by Tadasa (1977), who explained an experiment about the biodegradation pathway of the compounds by *Corynebacterium* sp. Some *Pseudomonas* and *Bacillus* also reported their capability to transform the compound into vanillin. *Pseudomonas resinovorans* SPR1, and *Bacillus safensis* SMS1003 could convert eugenol into vanillin and such derivative compounds (Ashengroph *et al.* 2011; Singh *et al.* 2018). In addition to bacteria, several fungi and yeast, such as *Aspergillus luchuensis* (Taira *et al.* 2018) and *Trichosporon asahii* (Ashengroph and Amini 2017) also reported their ability to produce vanillin from the compounds. Moreover, genes and enzymes involved in the route of eugenol catabolism to vanillin have also been reported (Figure 9) (Overhage *et al.* 2003).

The difference in vanillin concentrations obtained from each isolate was caused by its different pathways for degrading eugenol. In addition, the different enzyme activity of each isolate in transforming eugenol into vanillin also affected the concentration of the resulting product (Priefert *et al.* 2001). BKL 15 isolate was chosen because it produced the highest vanillin product compared to other isolates, which was 378.66 mg/L. Preliminary optimization of biotransformation parameters, including substrate (eugenol), yeast extract, casamino acid, and incubation time, were carried out to observe its effect on vanillin production by BKL 15 isolate. Optimization of the eugenol

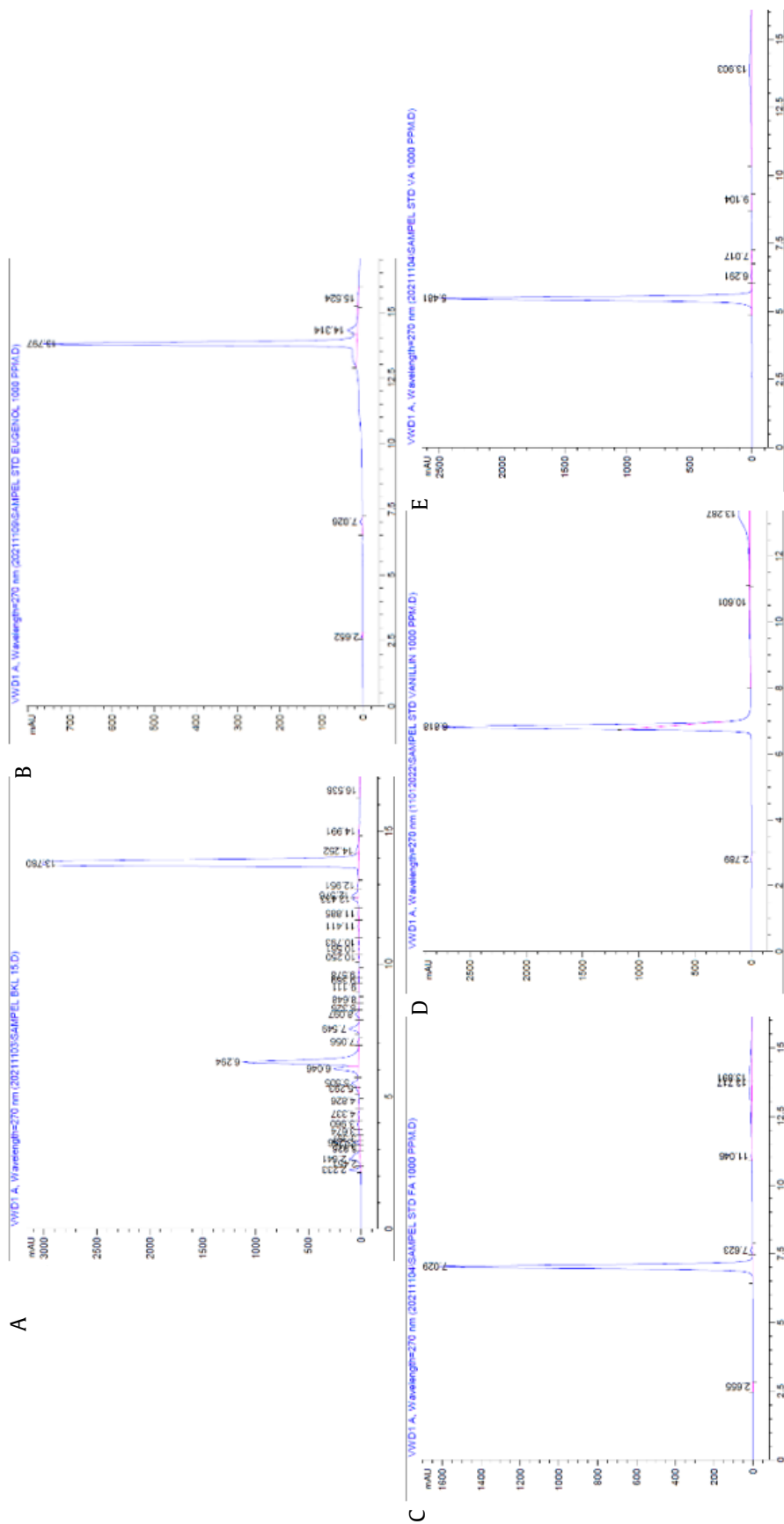


Figure 2. HPLC chromatogram profile of BKL 15 isolate (A), authentic standard eugenol (B), ferulic acid (C), vanillin (D), and vanillic acid (E) at a wavelength of 270 nm

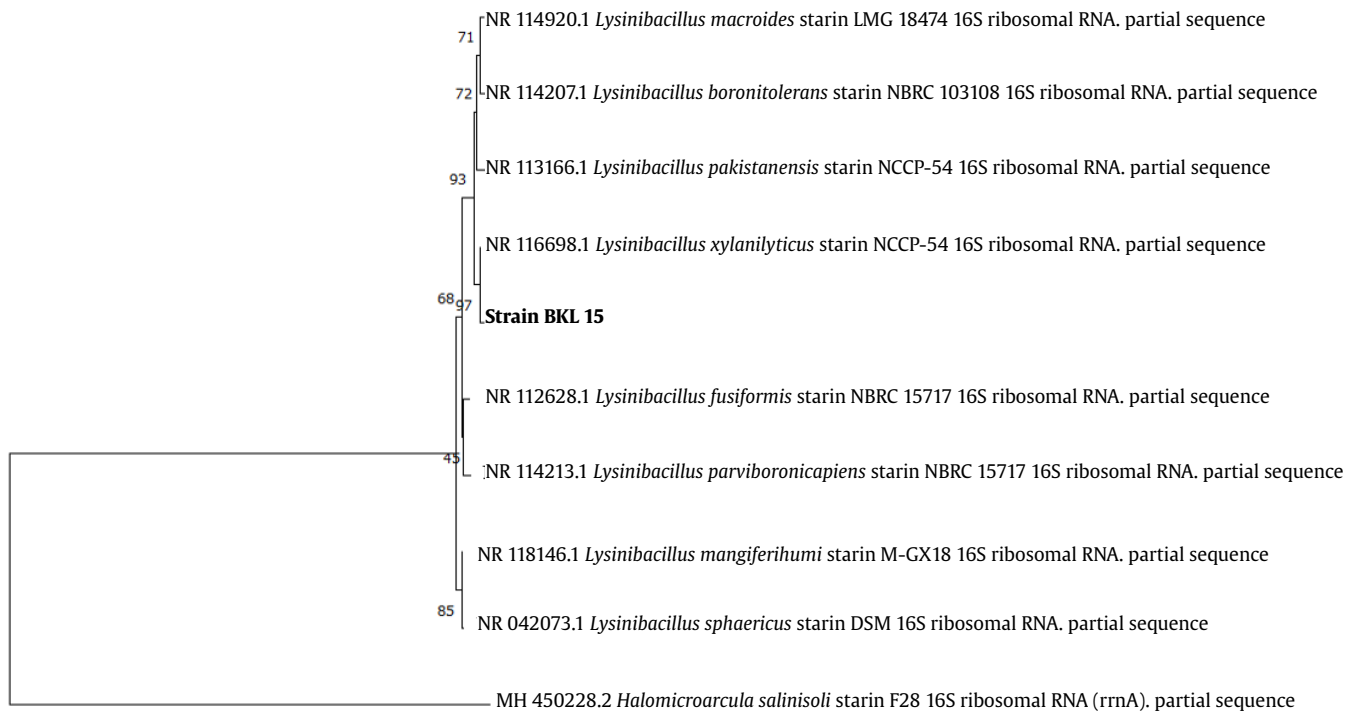


Figure 3. BKL 15 isolate phylogenetic tree based on 16S rRNA gene compared with its closely related species in GenBank

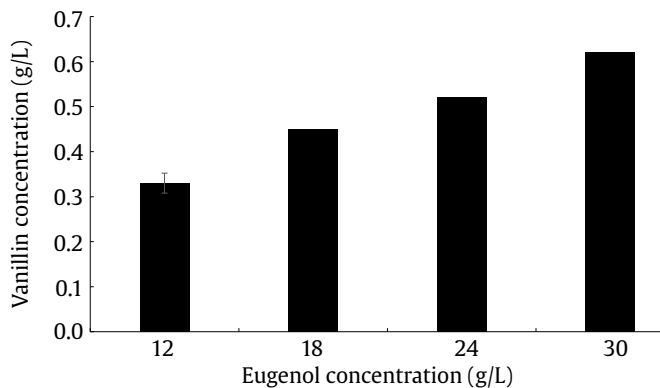


Figure 4. Vanillin concentration in crude extract of BKL 15 isolate grown in a medium supplemented with various concentrations of eugenol

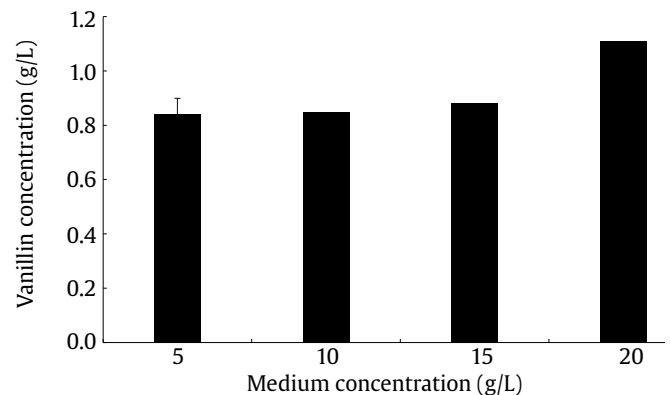


Figure 5. Vanillin concentration in crude extract of BKL 15 isolate in the grown medium based on variations in the concentration of yeast extract and cassamino acid

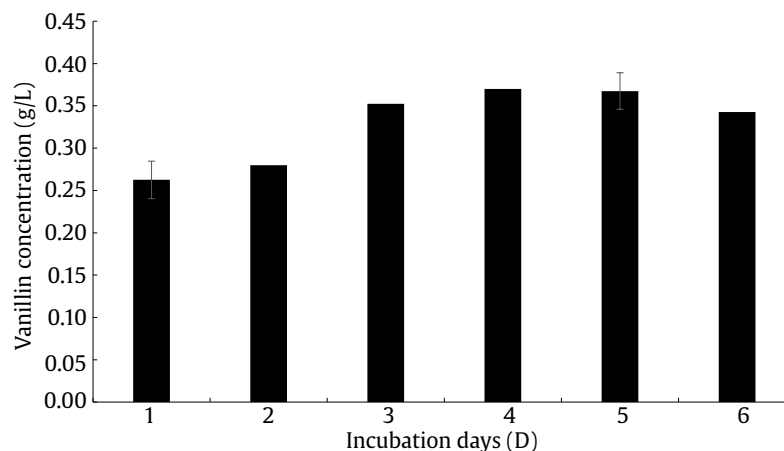


Figure 6. Vanillin concentration in crude extract of BKL 15 isolate produced at different incubation time

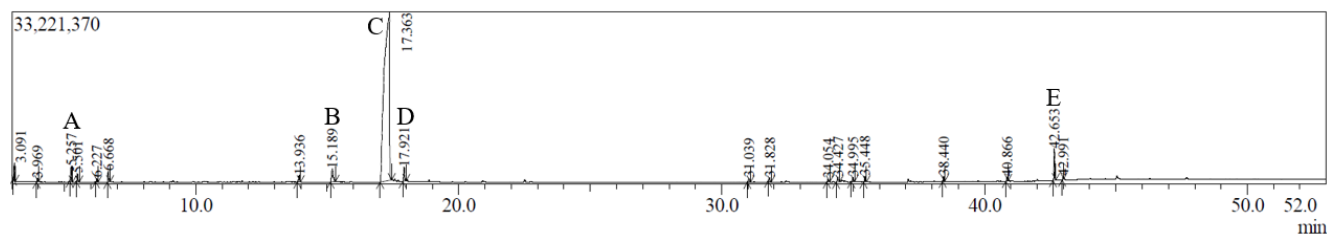


Figure 7. Chromatogram of GC-MS analysis of the crude extract of BKL 15 isolate. (A) ethylbenzene, (B) phenol 4-(2-propenyl)-, (C) eugenol, (D) vanillin, (E) bis(2-ethylhexyl) phthalates

Table 1. Compounds identified from crude extract of BKL 15 isolate through gas chromatography-mass spectrometry (GC-MS)

Peak	RT (min)	Compounds	Formula	Molecular weight (g/mol)	Similarity (%)	Area (%)
1	3.091	Toluene	C_7H_8	92	98	0.92
2	3.969	Acetic acid	$C_6H_{12}O_2$	116	97	0.23
3	5.257	Ethylbenzene	C_8H_{10}	106	98	1.26
4	5.501	Benzene, 1,3-dimethyl-	C_8H_{10}	106	98	0.72
5	6.227	o-Xylene	C_8H_{10}	106	98	0.28
6	6.668	Ethanol, 2-butoxy-	$C_6H_{14}O_2$	118	97	0.85
7	13.936	Methyl salicylate	$C_8H_8O_3$	152	90	0.64
8	15.19	Phenol 4-(2-propenyl)-	$C_9H_{10}O$	134	95	2.09
9	17.35	Eugenol	$C_{10}H_{12}O_2$	164	96	85.96
10	17.91	Vanillin	$C_8H_8O_3$	152	97	1.18
11	31.039	9-Eicosene, (E)-	$C_{20}H_{40}$	280	97	0.46
12	31.828	Cyclo(L-prolyl-L-valine)	$C_{10}H_{16}N_2O_2$	196	94	0.44
13	34.054	Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	$C_{11}H_{18}N_2O_2$	210	90	0.31
14	34.427	Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	$C_{11}H_{18}N_2O_2$	210	92	0.54
15	34.995	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	94	0.43
16	35.448	1-Octadecene	$C_{18}H_{36}$	252	96	0.48
17	38.440	1-Hexacosene	$C_{26}H_{52}$	364	97	0.33
18	40.866	1-Tetracosene	$C_{24}H_{48}$	336	98	0.27
19	42.65	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390	96	2.41
20	42.991	1-Hexacosene	$C_{26}H_{52}$	364	96	0.18

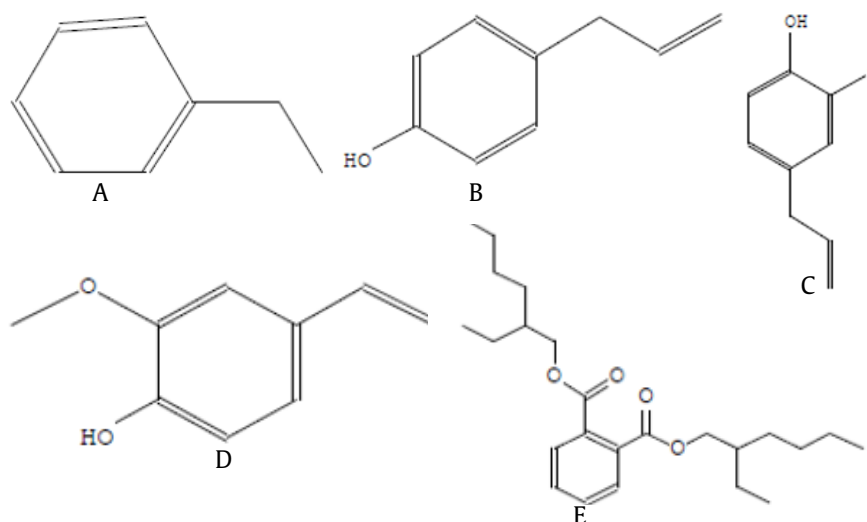


Figure 8. Chemical structure of the 5 compounds with the highest concentration. (A) ethylbenzene, (B) phenol 4-(2-propenyl)-, (C) eugenol, (D) vanillin, (E) bis(2-ethylhexyl) phthalates

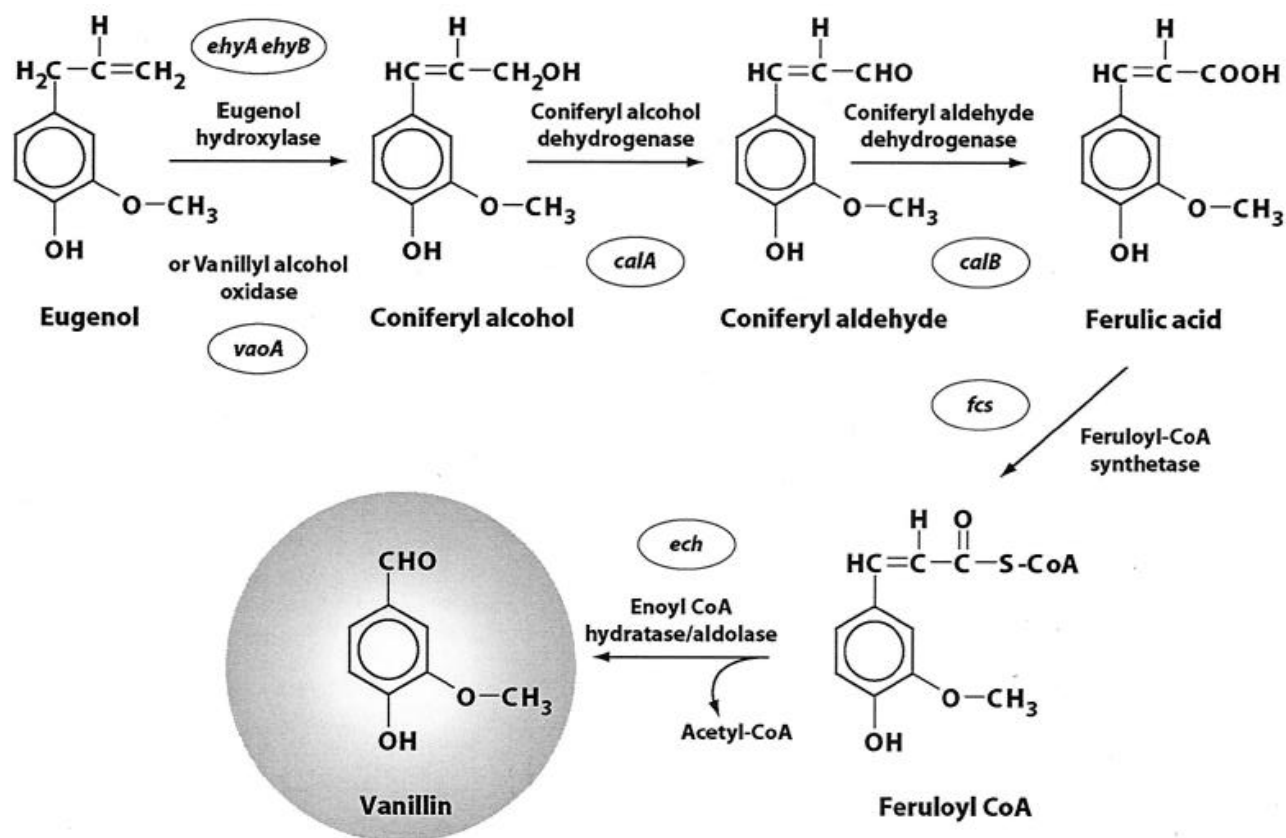


Figure 9. Bacterial biotransformation of eugenol to vanillin

substrate showed that at a concentration of 30 g/L eugenol, vanillin production reached 0.61 g/L (molar yield 2.23%). However, further increases in eugenol concentrations remain to be investigated. Several other reports have shown that higher concentrations of eugenol substrates will reduce the production of vanillin and other derived metabolites. A study by Singh *et al.* (2018) showed that the optimum eugenol concentration for the production of vanillin used was 500 mg/L, and there was a decrease after that. In addition, the optimum eugenol concentration was also reported to be at a concentration of 2.5 g/L, and vanillin production decreased after the addition of the substrate (Ashengroph *et al.* 2011). This was thought to be related to the nature of eugenol, which is a toxic compound with antibacterial properties (Nejad *et al.* 2017).

Based on medium optimization measurement, the concentration of yeast extract and casamino acid of 20 g/L was chosen as the optimum concentration in achieving the highest vanillin production in the optimization medium of 1.11 g/L (4.99 % molar yield). Yeast extract and casamino acid are common ingredients used to promote bacterial growth. Yeast

extract was well known to act as an effective vitamin as well as nitrogen. A study conducted by Hakobyan *et al.* (2012) proved that the addition of this ingredient to the *Rhodobacter sphaeroides* medium could increase cell growth and photoproduction of hydrogen by the bacterium strain isolated from mineral springs. In addition, another report also stated that yeast extract can increase the stability of biogenic tooeleite by the bacterium *Acidithiobacillus ferrooxidans*, thereby reducing highly toxic soluble arsenite (III) (Li *et al.* 2022).

Meanwhile, the addition of casamino acid to the *Desulfovibrio marinisediminis* medium played a role in increasing the growth of the sulfate-reducing bacteria (Takii *et al.* 2008). Optimization based on differences in incubation time was observed in the 1 to 6-day incubation period based on observations of the growth of isolates at the isolation stage. The optimum concentration was obtained on the fourth and fifth days with vanillin production of 0.37 g/L (molar yield 1.66%). The reduced production of vanillin on the sixth day was thought to be related to the accumulation of nutrients and eugenol in the growing medium, which

had been reduced. Moreover, we hypothesize that the decrease in vanillin production is also due to the toxicity of vanillin itself. Banerjee and Chattopadhyay (2019) stated that vanillin was a molecule that had a toxic effect on bacteria and was easily degraded at high concentrations. The toxicity of vanillin will inhibit bacterial cell growth and reduce the yield of vanillin (Kaur and Chakraborty 2013).

Twenty compounds have a peak area percentage of 0.18–85.96%, and 5 of those compounds have the highest concentration with a peak area of more than 1%. The five main compounds with the highest peak presentations were ethylbenzene, phenol 4-(2-propenyl)-, eugenol, vanillin, and bis(2-ethylhexyl) phthalate (Table 1). Eugenol and vanillin are the two main compounds targeted for metabolite detection in this study. The presence of other detected compounds indicated that not all of the eugenol substrates were transformed into vanillin. Various studies have been reported to increase the production of vanillin. *Escherichia coli* carrying the gene encoding the enzymes feruloyl-CoA synthetase and feruloyl-CoA hydratase/aldolase, as well as the activation of the gene encoding vanillin dehydrogenase from *Pseudomonas* sp. (Barghini *et al.* 2007; Gioia *et al.* 2011; Luziatelli *et al.* 2019). Biotransformation processes using adsorbent resins (Hua *et al.* 2007b), production using baker's yeast (Brochado *et al.* 2010), and mimicking a natural pathway of plants using microbial genes (Ni *et al.* 2015) are some other examples that have been developed to improve the vanillin production process.

The results of the molecular analysis of the 16S rRNA gene showed that the BKL 15 isolate was close to the *Lysinibacillus xylaniticus* strain XDB9 isolate (Figure 3). This genus is commonly found in soil. The bacterium is a group of Gram-positive, straight rods, forming endospores, cell size 0.8–1.0 × 3.0–5.0 μm, and motile. This bacterium can also grow at various temperatures (10–40°C) and pH five conditions and is able to utilize glucose, fructose, cellobiose, gluconate, 2-ketogluconate, and 5-ketogluconate as a source of nutrition (Lee *et al.* 2010). As far as information is concerned, our study is the first to report the ability of *L. xylaniticus* species to transform eugenol into vanillin.

The bacterial species *L. xylaniticus* was first reported by Lee *et al.* (2010), related to its ability to degrade xylan. Based on the structure of the peptidoglycan cell wall, the genus *Lysinibacillus* was proposed as a novel species by Ahmed *et al.* (2007). This bacterial genus was

the result of the reclassification of two *Bacillus* species based on polyphasic taxonomic studies. The ability of *Bacillus* species has been widely reported for its ability to produce vanillin through a biotransformation process using certain substrates. Several novel strains, such as *Bacillus pumilus* (Hua *et al.* 2007a) and *Bacillus subtilis* (Chen *et al.* 2016), reported their ability to produce vanillin using isoeugenol and ferulic acid as substrates. A study by Singh *et al.* (2018) also found a new strain, namely *Bacillus safensis*, and reported its ability to use eugenol as a substrate to produce vanillin.

Throughout the publications we have read, this is the first report of a vanillin-producing *L. xylanolyticus*. This study succeeded in exploring the novel bacterium species which have the potential to produce vanillin. Based on the information about the intrinsic characteristics of this species, it would be very interesting to conduct further studies to see its potential to produce vanillin directly from agricultural waste biomass, which is abundantly available in Indonesia. Furthermore, the possibility of a genetic engineering strategy for these wild-type strains also needs to be considered.

Conflict of Interest

The authors declare no conflict of interest.

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