BIODIVERSITAS
Volume 22, Number 8, August 2021
Pages: 3156-3162

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d220809

Isolation, identification, and analysis of the invertase-producing bacteria abundance in sugarcane rhizosphere soil with different plant productivity levels

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Manuscript received: 25 March 2021. Revision accepted: 11 July 2021.

Abstract. *Lase ERK, Giyanto Santosa DA. 2021. Isolation, identification, and analysis of the invertase-producing bacteria abundance in sugarcane rhizosphere soil with different plant productivity levels. Biodiversitas 22: 3156-3162.* microbes and enzymes play an important role in maintaining the stability of soil ecosystems. Invertase is one of the soil enzymes produced by microbes to hydrolyze sucrose in the environment. The aims of this study were to investigate the abundance of invertase-producing bacteria and soil invertase activity in sugarcane rhizosphere soil with different plant productivity levels and their correlation with soil chemical and physical properties and to obtain invertase-producing bacteria from the sugarcane rhizosphere. The samples of sugarcane rhizosphere soil were collected using a randomized sampling method and determine their physical-chemical properties, the abundance of invertase-producing bacteria, and soil invertase activity. Twenty invertase-producing bacteria were successfully isolated and tested their abilities to produce invertase qualitatively and quantitatively. The three best isolates (ScT112, ScT124, and ScR301) were molecularly identified using the 16S rRNA gene and phylogenetic tree analysis. The results of this study indicate that there is a significant correlation between invertase bacterial abundance and soil invertase activity. The abundance of invertase-producing bacteria correlates with pH, organic C, total N, and soil sand content. Invertase activity in the soil correlates with pH and organic C. Based on the phylogenetic tree, the isolates of ScT112 had the closest homology with *Cupriavidus* sp., ScT124 was homologous with *Klebsiella variicola*, while ScR301 was homologous with *Pantoea* sp. These three isolates have the potential to be developed in industrial biotechnology to produce invertase. The hydrolysis zones of ScT112, ScT124, and ScR301 were 3.12 cm, 2.76 cm, and 2.55 cm, respectively with the invertase activity of 27.82 U mL^{-1}, 24.56 U $mL^{-1,}$ and 24.08 U mL^{-1}.

Keywords: 16S rRNA, invertase-producing bacteria, soil invertase activity, sucrose

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is one of the agricultural commodities that play an important role in maintaining the production of the sugar industry. In recent years, sugar consumption in Indonesia has increased sharply, but it is not accompanied by increased sugar production. In 2017, national sugar production decreased by 7.28% compared to 2016, which was recorded at 2.19 million tons (BPS, 2018). The Indonesian government strives for sugar self-sufficiency as one of the steps towards national food security. Various studies aimed at developing and elevating sugarcane productivity are imperative to be evaluated. Soil microorganisms and soil enzymes are biological indicators of soil health and fertility, are involved in many important soil biological processes, such as nutrient mineralization and cycling, soil organic matter transformation, and residue decomposition. Biochemical processes carried out by soil microorganisms are critical to maintaining soil quality and sustainability (Senechkin et al. 2014; Bruggen et al. 2015).

Invertase is one type of soil enzyme that describes soil microorganisms' activity and the intensity of carbon metabolism because it is associated with the soil carbon cycle (Lagomarsino et al. 2009; Paz-Ferreiro et al. 2011). Soil biochemical properties were significantly correlated with soil microorganism communities. The higher abundance of beneficial microbes is positively related to the higher soil quality, including better plant growth, lower disease incidence, higher nutrient contents, soil enzyme activities, and soil pH (Wang et al. 2017). Soil management practices have a direct effect on soil microbial communities and enzymes. Many factors affect soil microbial communities under continuous cropping, including characteristics and environmental conditions. The relationship between the abundance of invertase-producing bacteria and soil invertase activity in the sugarcane rhizosphere with different plant productivity levels is still unknown. Their correlation to soil chemical and physical properties need to be studied further.

Apart from being known as a soil enzyme, invertase is also widely used in the food industry because of its hydrolyze sucrose ability. Invertase is an enzyme commonly used in the food industry because it produces inverted sugars that crystallized faster and sweeter taste (Kulshrestha et al. 2013). Some isolates of invertase-

producing bacteria include *Bacillus cereus* TA-11 (Yoon et al. 2007), *Arthrobacter* sp. (Win et al. 2004; Xu et al. 2009), and *Streptomyces* sp. (Kaur and Sharma, 2005) isolated from the rhizosphere. The rhizosphere soil is the soil around plant roots that affected directly by many root exudates as an energy source for microorganisms (Bobille et al. 2016).

Information and data on invertase from bacteria are still very limited (Lincoln and More, 2017). The exploration of invertase-producing bacteria from the ecosystem in Indonesia is still very limited. Therefore the potential for the discovery of invertase-producing bacteria for development in the field of biotechnology is enormous. It is due to Indonesia is a country with the highest biodiversity of flora, fauna, and microorganisms. It is necessary to evaluate the abundance of invertase-producing bacteria and soil invertase activity in the sugarcane rhizosphere with different productivity levels. The data could support the sustainability of sugarcane productivity.

MATERIALS AND METHODS

Study area

The study site was located in PT. Rajawali II Sugarcane plantation, Jatitujuh, Majalengka (108°6'3"-108°16'24" E, 6°31'2"-6°36'40" S), West Java, Indonesia. The fields have a subtropical humid climate (an annual rainfall of 1500-2500 mm and mean annual temperature of 26.3-27.1 °C) where sugarcane has been cultivated continuously for more than 30 years. Soil samples were taken from the sugarcane rhizosphere and determined based on the level of crop productivity. The categorization of sugarcane productivity levels is as follows: high productivity (> 60 tons/ha), medium productivity (40-60 tons/ha), and low productivity (< 40 tons/ha) of ten months of planting. The sugarcane in this location is *ratoon 1* from Kidang Kencana (KK) variety. Soil samples of each level of productivity were taken from six different locations (Figure 1). At each location (4 ha), the composite sample was collected from a mixture of 9 random soil cores (0-20 cm depth). Each soil sample was partitioned into two sub-samples, one for biological properties analysis and another for analysis of physical and chemical properties after air-dried.

Procedures

Analysis of soil biological, physical, and chemical properties

The analysis of soil biological properties in this study included the abundance of invertase-producing bacteria and soil invertase activity. The abundance of invertase-producing bacteria was carried out using the *Total Plate Count* (TPC) method by using selective media of sucrose hydrolysis (Reddy et al. 2010). Soil invertase activity was analyzed by quantitative methods described by Schinner and Mersi (1990). The analysis of chemical and physical properties of soil included pH, organic C, total N, available P, available K, CEC, and soil texture.

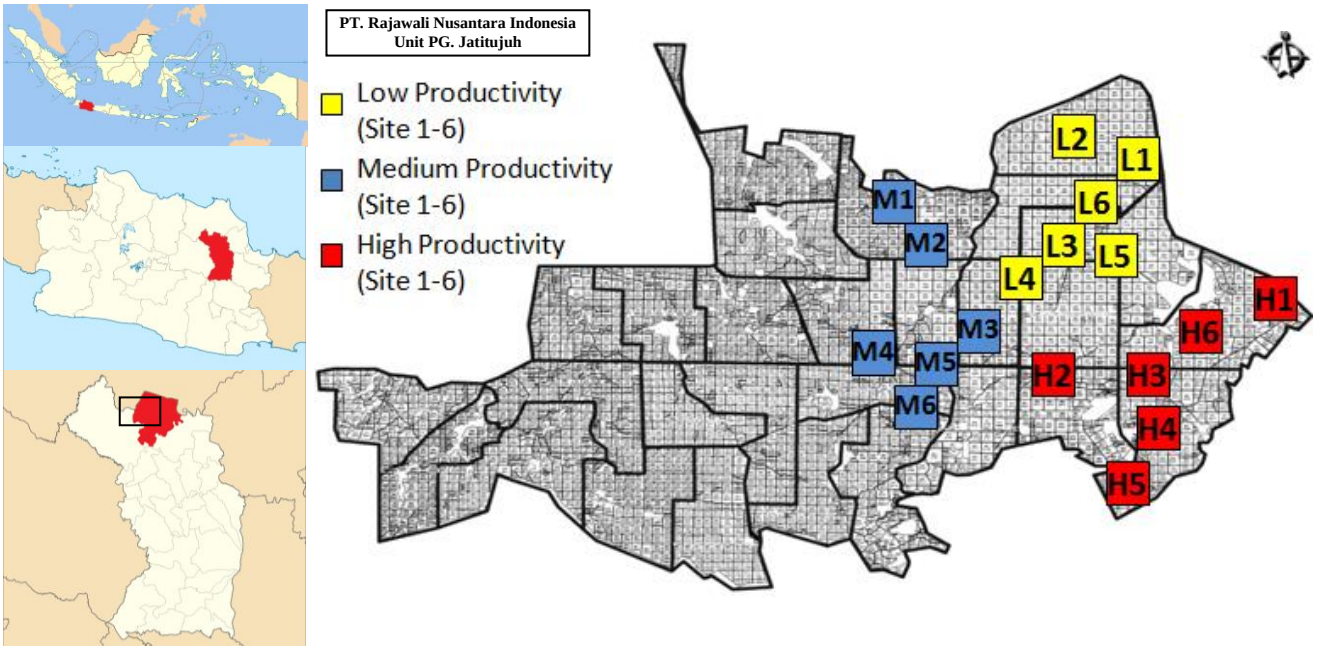

Figure 1. Sampling sites and map of PT. Rajawali II Sugarcane Plantation, Jatitujuh, Majalengka, West Java, Indonesia

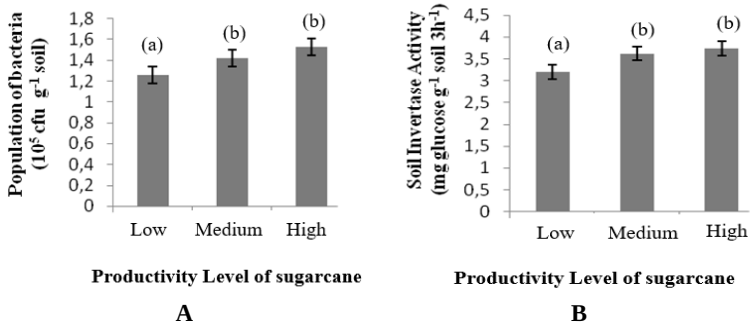

Figure 2. Soil invertase-producing bacteria abundance (A) and invertase activity (B) under low, medium, and high productivity levels of sugarcane. Vertical T bars indicate standard deviations. Bars with different letters indicate significant differences ($P < 0.05$)

Isolation, selection, and identification of invertase-producing bacteria

Invertase-producing bacteria were isolated from the sugarcane rhizosphere. The sampling method was the purposive method. The selection of invertase-producing bacteria was carried out by analyzing the qualitative and quantitative activity of invertase by each bacteria isolate. The qualitative analysis was determined by *Radial Diffusion Method* (Reddy et al. 2010), while the quantitative analysis was tested by DNS (*dinitrosalicylic acid*) reagent (Miller, 1959). Molecular identification of selected isolates was carried out in several stages: (1) isolation of bacterial genomic DNA by referring to the protocol of *PrestoTM gDNA Bacteria Mini Kit* (Geneaid). The bacterial genomic DNA purity was analyzed by *NanoDrop Spectrophotometer*; (2) amplification of 16S rRNA gene from bacterial isolates by referring to the *My Taq™ Red Mix* (Bioline) protocol. The PCR results were verified by electrophoresis on 1% agarose gel; (3) DNA sequencing and phylogenetic tree analysis. The DNA sequences of invertase-producing bacteria compared to DNA sequences of other bacteria strains in the database using the BLAST-N program available on the NCBI website (*National Center for Biotechnology*). Phylogenetic analysis is carried out using the MEGA 6 program.

Data analysis

Analysis of variance (ANOVA) and Pearson's correlation analysis was carried out with SPSS (version 16.0). The significance of the parameters was tested using the least significant difference multiple range test at $P < 0.05$ after one-way ANOVA.

RESULTS AND DISCUSSION

The abundance of invertase-producing bacteria and soil invertase activity

The abundance of invertase-producing bacteria and soil invertase activity in the sugarcane rhizosphere with different productivity was presented in Figure 2. The population density of invertase-producing bacteria in the sugarcane rhizosphere ranged from 1.26×10^5-1.53×10^5 CFU g^{-1} soil, while the invertase activity of bacteria collected from the sugarcane rhizosphere ranged 3.20-3.74 mg glucose g^{-1} soil $3h^{-1}$. The productivity level of sugarcane is directly proportional to the abundance of invertase-producing bacteria and soil invertase activity. The abundance of invertase-producing bacteria and invertase activity in the sugarcane rhizosphere with different levels of productivity were influenced by biotic and abiotic environmental factors, especially root exudates and soil organic matter content.

Analysis of soil physical and chemical properties and its correlation with the abundance of invertase bacteria and soil invertase activity

The physical-chemical properties of soil in sugarcane fields with high productivity are relatively better compared to medium and low productivity levels (Table 1). The pH, organic C, and available P of soil were significantly ($P < 0.05$) higher in sugarcane fields with a high productivity level than that of low and medium productivity. The soil texture at the study site was sandy loam and the soil pH tends to be slightly acidic.

The relationship between several biological, chemical, and physical properties of the soil was carried out by correlation analysis (Table 2). The abundance of invertase-producing bacteria positively correlated invertase activity, pH, organic C, and total N of soil, but negatively correlated with soil sand content. Invertase activity positively correlated with the abundance of invertase-producing bacteria, pH, and organic C content of the soil. Invertase can decompose complex organic compounds into subunits assimilated by microbes, leading to delayed changes of microbiological parameters.

Isolation, selection, and identification of invertase-producing bacteria

Twenty strains of invertase-producing bacteria have been isolated from the sugarcane rhizosphere which consisted of 11 Gram-positive and 9 Gram-negative bacteria. The isolates were analyzed for their ability to produce extracellular invertase enzymes qualitatively and quantitatively. The qualitative invertase activity analysis showed that the range of hydrolysis zone produced by bacterial isolates was 0.11-3.10 cm (Figure 3). Three isolates with large hydrolysis zone diameters were ScT112 (3.12 cm), ScT124 (2.76 cm), and ScR301(2.55 cm). The isolate with the lowest hydrolytic activity was ScT109 (0.11 cm).

Table 1. The physical and chemical properties of soil in sugarcane fields with different levels of productivity

Parameters	Productivity level of sugarcane		
	Low	Medium	High
pH H_2O	5.67 ± 0.07[a]	5.92 ± 0.09[b]	6.06 ± 0.11[b]
Organic C (%)	1.78 ± 0.06[a]	1.88 ± 0.07[a]	2.20 ± 0.04[b]
Total N (%)	0.18 ± 0.08[a]	0.19 ± 0.01[a]	0.21 ± 0.01[a]
Available P (P_2O_5 ppm)	49.15 ± 1.41[a]	48.71 ± 1.16[a]	54.31 ± 1.48[b]
Available K (cmol (+) kg^{-1})	0.54 ± 0.07[a]	0.57 ± 0.05[b]	0.58 ± 0.01[b]
CEC (cmol (+) kg^{-1})	16.40 ± 0.44[a]	19.20 ± 0.73[b]	19.04 ± 1.04[b]
Clay (%)	30.61 ± 0.23[a]	31.35 ± 0.50[a]	31.88 ± 0.72[a]
Sand (%)	43.93 ± 0.30[a]	42.40 ± 0.30[b]	42.79 ± 0.43[b]
Silt (%)	25.45 ± 0.42[a]	26.24 ± 0.40[a]	25.32 ± 0.55[a]

Note: Data are mean values with standard errors (SE). Data within a row followed by different letters indicate a significant ($P < 0.05$) difference

Table 3. Invertase activity of ten selected bacterial isolates

Strains	Analysis of invertase activity	
	Qualitative Test (cm)	Quantitative Test (U/mL)
ScT112	3.12 ± 0.06	27.82 ± 0.84
ScT124	2.76 ± 0.04	24.56 ± 1.78
ScR301	2.55 ± 0.06	24.08 ± 2.21
ScT105	2.16 ± 0.08	22.84 ± 0.91
Sc207	1.57 ± 0.06	19.13 ± 1.60
ScT122	1.28 ± 0.04	13.12 ± 2.28
ScT103	1.26 ± 0.33	14.46 ± 1.28
Sc211	1.13 ± 0.08	14.78 ± 0.33
ScT129	0.94 ± 0.86	11.53 ± 1.66
Sc209	0.93 ± 0.10	12.17 ± 1.38

Table 2. Pearson′s correlation coefficients among measured parameters

	BA	pH	OC	TN	AP	AK	CEC	C	SN	SI
pH	0.479*									
OC	0.609**	0.535*								
TN	0.581*	0.484*	0.545*							
AP	0.398	0.400	0.596**	0.244						
AK	0.341	0.213	0.413	0.137	0.285					
CEC	0.426	0.324	0.249	0.317	0.172	0.516*				
C	-0.306	0.246	0.395	0.181	0.462	0.325	0.286			
SN	-0.508*	-0.220	-0.192	-0.091	-0.274	-0.465	-0.463	-0.552*		
SL	0.111	-0.083	-0.281	-0.126	-0.284	0.049	0.092	-0.650**	-0.275	
IA	0.644**	0.591**	0.594**	0.152	0.427	0.421	0.460	0.442	-0.456	-0.094

Note: BA: invertase-producing bacteria abundance, pH: soil pH, OC: soil organic carbon, TN: soil total nitrogen, PA: available phosphorus, AK: available potassium, CEC: cations exchange capacity, C: clay content (%), SN: sand content (%), SL: silt content (%), IA: soil invertase activity; *significant at 5% level, ** significant at 1% level

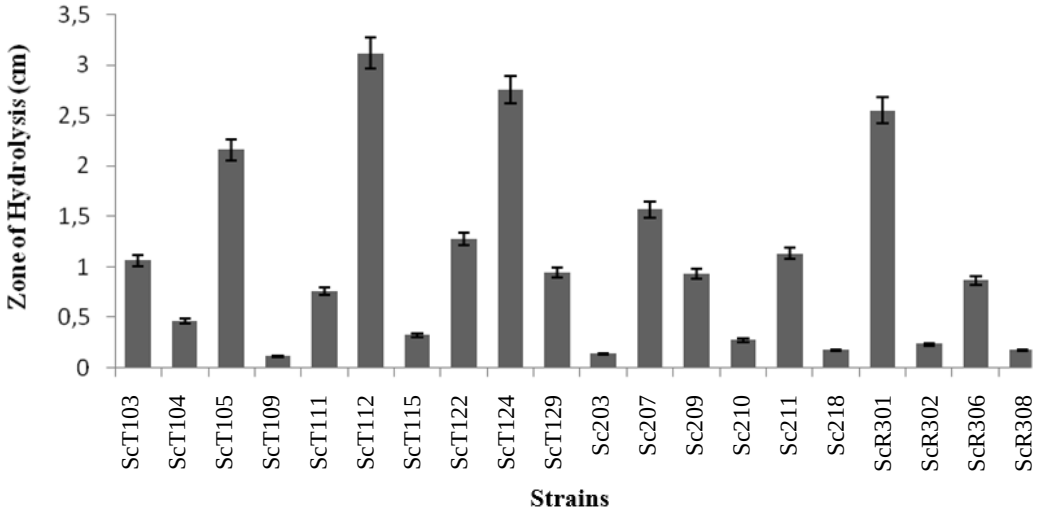

Figure 3. Diameter of hydrolysis zone produced by 20 isolates of invertase-producing bacteria isolated from the sugarcane rhizosphere

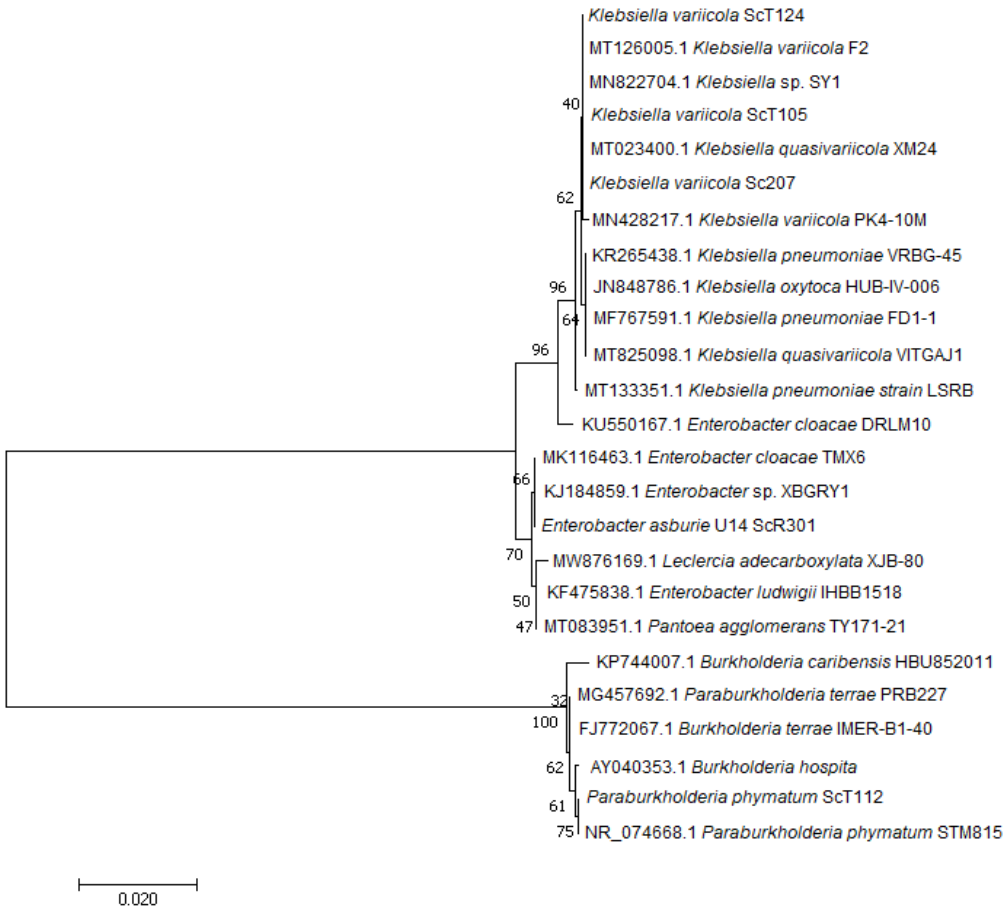

Figure 4. Phylogenetic tree of selected bacterial isolates: *Paraburkholderia phymatum* ScT112, *Enterobacter asburie* ScR301, *Klebsiella variicola* ScT124 based on 16s rRNA

Ten isolates with a high hydrolysis zone were tested for their ability in producing invertase enzymes quantitatively. The activity of invertase of ten bacterial isolates varied from 11.53-27.83 U/mL crude enzyme extract (Table 3). The three best isolates were ScT112 (27.82 U/mL, ScT124 (24.56 U/mL), and ScR301 (24.08 U/mL) were molecularly identified by 16S rRNA gene analysis. The results of 16S rRNA gene sequences and phylogenetic analysis (Figure 4), showed that ScT112 isolates were homologous with *Paraburkholderia phymatum* strain HBU08166 (99.15%), ScT124 isolates had the closest homology with *Klebsiella variicola* isolate F2 (100%), and ScR301 isolates had the closest homology to *Enterobacter asburiae* strain U4 (99.72%).

Discussion

The difference in abundance of invertase-producing bacteria and soil invertase activity in the sugarcane rhizosphere with different levels of productivity was caused by differences in the quality and quantity of root exudates and soil organic matter content. Lin *et al.* (2013) reported different types of expressed proteins in the sugarcane's rhizosphere with high and low productivity. These proteins are associated with the release of plant root exudates functioning as chemical compounds to attract certain bacteria towards roots. The content of soil organic matter also has a significant effect on the abundance of soil bacteria due to its important function as source nutrients and a substrate for soil microorganisms to increase activity and growth (Wang et al. 2019).

Soil organic carbon content in high productivity of sugarcane field was significantly higher than medium and low productivity. Higher organic C in high productivity fields was caused by the addition of organic fertilizer, compost, on the field with high productivity. According to Hartono *et al.* (2016), sugarcane plants that fertilized with compost have significantly better several growth parameters compared to sugarcane plants without the addition of compost. Increasing organic matter content in the soil has a positive effect on improving the quality and ability of the soil to provide nutrients to support plant growth and soil microbes. The soil texture at the study site was sandy loam. Soil texture and soil organic matter content influence the cation exchange capacity and soil ability to retain water and provide nutrients (Nath 2014).

Soil invertase activity was reported positively correlated with soil organic carbon content and soil microbial biomass because the components of the enzyme itself are mainly produced by microbes (Xiao et al. 2015). Invertase is extracellular enzymes produced by soil

microorganisms such as bacteria, fungi, and actinomycetes. Invertase activity in the soil is related to the degradation process of sucrose which is commonly found in plant tissue. The results of sucrose decomposition in the form of glucose and fructose are used by plants and other microorganisms as sources of carbon and energy (Frankenberger and Johanson, 1983).

Several studies have reported similar results that soil invertase positively correlated with pH and soil organic carbon content (Shi et al. 2008; Hu et al. 2013). The optimum pH value of some extracellular invertase enzymes from bacteria is in the pH range 5.5-7.6 (Lincoln and More, 2017). The enzyme activity decreases with increasing or decreasing pH of the environment exceeding the optimum pH value. Soil texture also affects the microbial abundance and mineralization processes in the soil. According to Hamarashid *et al.* (2010), the ability of the soil to provide organic material and total nitrogen in clay and sill particles is greater than the sand fraction. It affects the soil microbe activity and community structure and carbon mineralization processes.

A total of 20 isolates of invertase-producing bacteria were isolated from the sugarcane rhizosphere. The three best isolates with high qualitative and quantitative invertase activity were ScT112, ScT124, and ScR301. The three isolates were able to produce hydrolysis zones of 3.12 cm, 2.76 cm, and 2.55 cm, respectively. In media containing reducing sugars, *Triphenyl tetrazolium chloride* (TTC) would be reduced to *triphenylformazon* which was red and insoluble in water. The amount of formazan formed was proportional to the amount of reducing sugar in the media. The larger the diameter of the red zone indicated the higher the reducing sugar produced. Belorkar *et al.* (2015) analyzed sucrose hydrolysis activity from several fungi, yeast, and bacterial isolates by the same method and showed that bacterial groups had diameters ranging from 0.12-0.54 cm. The invertase activity produced by three isolates in this study was 27.82 U/mL, 24.56 U/mL, and 24.08 U/mL crude enzyme extract. Optimization of substrate fermentation, purification, and immobilization of enzyme was expected to significantly increase enzyme activity. The type of microbes that are intensively developed for invertase production in the industry mostly comes from the yeast and fungi groups, while the invertase from bacteria is still very limited.

Based on the similarity of 16S rRNA gene sequences and phylogenetic analysis, ScT112 isolates were homologous with *P. phymatum* strain HBU08166 (99.15%), ScT124 isolates had the closest homology with *K. variicola* isolate F2 (100%), and ScR301 isolates had the closest homology to *E. asburiae* strain U4 (99.72%). *P. phymatum*, *K. variicola,* and *E. asburiae* are several species of bacteria that have been previously reported and isolated from the sugarcane rhizosphere (Antwerpen et al. 2002; Magnani et al. 2010; Jeon et al. 2021). *K. variicola,* and *E. asburiae* were part of ordo Enterobacterales, and family Enterobacteriaceae. More than 90% of the wild-type strain of *Klebsiella* was Scr^+ could produce invertase. Genes scrA and scrB genes in *Klebsiella pneumoniae*, play a role in sucrose hydrolysis (Titgemeyer et al. 1996). The genus *Enterobacter* is a bacterium that is often found in the rhizosphere of plants, and most of them were reported to degrade sucrose (Khalifa et al. 2016; Chi et al. 2018). According to Wu and Birch (2005), *Pantoea dispersa*, as a part of ordo Enterobacterales, also produces highly efficient sucrose isomerase that can carry out two catalytic processes, hydrolysis, and isomerization of sucrose, with the final results of glucose and fructose in a ratio of 1: 1.

In conclusion, we found that the abundance of invertase-producing bacteria and invertase activity in the sugarcane rhizosphere varied at different levels of productivity. The abundance of invertase-producing bacteria is positively correlated with soil invertase activity, pH, C-organic, N-total, and negatively correlated with soil sand content. Soil invertase activity positively correlated with pH and organic C. Understanding the biological processes of crop become one of the considerations in making decisions to achieve sustainable agricultural land use. However, soil biology is a complex field of study, and research continues to reveal new findings related to the organism itself and the processes and factors that influence it. The discovery of invertase-producing bacteria (*P. phymatum*, *K. variicola*, and *E. asburiae*) from sugarcane rhizosphere in Indonesian ecosystems is important information about invertase-producing bacteria strains that can be used as genetic resources for further development of invertase enzymes.

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