

# Bioteknologi

## Biotechnological Studies

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### Chapter in book:

Webb CO, Cannon CH, Davies SJ. 2008. Ecological organization, biogeography, and the phylogenetic structure of rainforest tree communities. In: Carson W, Schnitzer S (eds) *Tropical Forest Community Ecology*. Wiley-Blackwell, New York.

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Assaeed AM. 2007. Seed production and dispersal of *Rhazya stricta*. 50<sup>th</sup> Annual Symposium of the International Association for Vegetation Science, Swansea, UK, 23-27 July 2007.

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Alikodra HS. 2000. Biodiversity for development of local autonomous government. In: Setyawan AD, Sutarno (eds.) *Toward Mount Lawu National Park; Proceeding of National Seminary and Workshop on Biodiversity Conservation to Protect and Save Germplasm in Java Island*. Universitas Sebelas Maret, Surakarta, 17-20 July 2000. [Indonesian]

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Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic *Escherichia coli* predator-prey ecosystem. *Mol Syst Biol* 4: 187. [www.molecularsystemsbiology.com](http://www.molecularsystemsbiology.com). DOI:10.1038/msb.2008.24

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# Antifungal evaluation of leaf extracts and fungicide against *Fusarium oxysporum* f.sp. *lycopersici* causal agent wilt of tomato

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**Abstract.** Osman AOA, Mohamed IS. 2017. Antifungal evaluation of leaf extracts and fungicide against *Fusarium oxysporum* f.sp. *lycopersici* causal agent wilt of tomato. *Bioteknologi* 14: 1-8. *Fusarium*-wilt can attack tomato plants, and it is considered a crop threatening disease of the worldwide. Research was conducted to study the anti-fungal activity of various aqueous leaf extracts, i.e., peppermint, sweet basil plants and river red gum, beside fungicide Revus Top® on the growth of the fungi *Fusarium oxysporum* f.sp. *lycopersici*, a causal agent of wilt in tomato. In addition to an untreated-control, the experiment took three levels concentration for each aqueous plant extracts, i.e., 25, 50 and 100% and fungicide and applied to tomato plants. Their inhibitory effects were investigated in terms of retarding the fungal growth percentage. The results revealed that despite the inhibitory effect of fungicide against the fungal growth was more dominant, which range from 83.2% to 100% (no growth was recorded), all levels of concentration of aqueous leaf extracts of the three test plants significantly inhibited the growth of *Fusarium* test compared to the control treatment. Over the course of the experiment, aqueous extracts of river red gum exposed relatively high inhibition zone (44.1, 53.1 and 53.1%) followed by sweet basil (36.8, 51.5, and 54.4%) and peppermint aqueous extract as well (35.5, 39.6 and 39.6%), respectively. There is a common preference that the highest concentration of the plant extracts (peppermint, sweet basil, and river red gum 100%) and fungicide (100%) gave the significantly highest inhibition zones percent (41.9%, 48.5%, 39.3%, and 99.3%) respectively compared to the untreated control. The results showed that the increase of the dosage of aqueous leaf extracts would consistently increase their antifungal activity. Apparently, the test fungus differs in its response to the different concentrations; but overall, growth inhibition increased with the concentration. The study results may be considered promising and serve to encourage others to carry out a phytochemicals analysis of different parts of river red gum plant using different solvents so to determine the bioactive ingredient in each of these parts.

**Keywords:** Antifungal, plants extracts, fungicide, *Fusarium oxysporum*, tomato wilt

## INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.), a member of the family Solanaceae, is an important crop plant in providing human nutrition. The plant is considered to have originated in South America, and it distributed to Europe in the 16th century, before being distributed around the globe (Rick 1977). Solanaceae members are important cultivated-crop plants that contain essential amino acid, vitamins, and minerals (Sainju et al. 2003). Tomatoes are rich in vitamin C content (Kanyomeka and Shivute 2005) and antioxidant substances, such as lycopene. The tomato is also believed to be an important fruit for preventing and combating cancers (Agarwal and Rao 2000). With a pleasant flavor, high nutrition value, easy to cultivate, and the ability to fruit in a range of environments, the tomato is understandably a popular vegetable plant in many countries. The estimated global annual production of tomato is 95 million Mt (FAO 2002).

Tomato cultivation takes place in Sudan, with tomato farms occupying an area greater than 36540 hectares. With good irrigation, the area has supplied tons of tomato per hectare per year and provides significant benefits for local consumption and export purposes (AOAD 2007; Dawelbeit et al. 2010).

The promising value of tomato can be hampered by the fact that the crop is threatened by several diseases. This can be a negative impact on the growth and the total production worldwide. Among these, the wilt caused by pathogenic fungi, *Fusarium*, remains a challenging task in terms of best management to overcome the disease (Rick 1976; Agrios 2000; Srinon et al. 2006). *Fusarium oxysporum* f.sp. *lycopersici* (Sacc.) W. is a fungus that specifically infects the tomato plant and has caused a significant loss of the global production yield (Rick 1979; Cal et al. 2004; Srinon et al. 2006).

In Sudan, among the diseases known limiting the production of tomato, the wilt caused by *Fusarium oxysporum* f.sp. *lycopersici* is the most affecting one (Bhatia et al. 2004). Previous reports indicated that the disease is more severe for tomato planted in the traditional production areas characterized by the planting system on which the plant is grown on stored soil moisture after the flood waters of the Nile River subsides.

*Fusarium* wilt of tomato has been managed primarily using resistant varieties (Jalali and Chand 1992), but a breakdown in resistance of these varieties due to the evolution of virulent races of the pathogen have undermined their importance in recent years (Haware and Nene 1982; Jiménez-Díaz et al. 1993). In fact, numerous strategies have been proposed to control this fungal

pathogen (Biondi et al. 2004; Ahmed 2011). Methods like solarization, disinfection, seed treatment with synthetic fungicides, crop rotation and mixed cropping were also in use (Sullivan 2004). However, management of seed-borne and soil-borne diseases such as wilt, caused by *Fusarium* species has remained problematic (Haware and Kannaiyan 1992; Rao and Balachadran 2002).

Recently, many efforts by recognizing antimicrobial compounds in higher plants, gave promising strategies in combating plant pathogenic diseases. Biodegradability and selective in the toxicity of such compounds are considered valuable properties for controlling some plant diseases (Schmutterer 2002).

This study was undertaken to focus on the investigation of the potentiality of higher plant extracts and synthetic fungicides for management of *Fusarium* wilt of tomato caused by *Fusarium oxysporum* f.sp. *lycopersici*. This was done under laboratory conditions, to formulate a promising disease management approach with the following objectives: (i) To explore the antifungal potential of some higher plants aqueous crude extract against *F. oxysporum* f.sp. *lycopersici*. (ii) To evaluate the effect of systemic fungicide on fungal growth. (iii) To develop promising disease control components against *Fusarium* wilt of tomato.

## MATERIALS AND METHODS

### Experimental site

This study was conducted under laboratory conditions at Plant Pathology Department, College of Agricultural Studies "Shambat", Sudan University of Science and Technology (SUST) within the period January to March 2015. The study focused on evaluating the antifungal activity of sweet basil, peppermint, and River red gum (Table 1) leaves aqueous extracts and efficacy of fungicide, Revus Top<sup>®</sup>, against *Fusarium oxysporum* f.sp. *lycopersici*.

### Fungal inoculum

Random samples were collected from roots and stems of infected tomatoes plants (*Lycopersicon esculentum* Mill.) in fields at Wad RamLi area. Secured samples were put in paper bags and brought to the laboratory, where they were kept in the refrigerator for further investigations.

### Isolation of the pathogen from tomato

Isolation was done from diseased roots and stem of infected tomato plants showing typical symptoms of *Fusarium* wilts. They were then cut into pieces of 0.5 to 1 cm and washed under tap water for about 5 minutes to remove soil particles. The washed pieces were dipped in 70% ethyl alcohol (5% concentration) for 2 minutes and rinsed three times in sterilized distilled water and dried on sterilized filter paper. The sterilized sections were then plated at the rate of 6 sections per plate on potato dextrose agar (PDA) medium.

**Table 1.** List of plant species tested for antifungal activity.

Name of plant	Family
Sweet basil ( <i>Ocimum basilicum</i> L.)	Lamiaceae
Peppermint ( <i>Mentha piperita</i> L.)	Lamiaceae
River red gum ( <i>Eucalyptus camaldulensis</i> Dehnh.)	Myrtaceae

### Identification of pathogen

The Petri dishes were incubated at 25°C. After incubation for 7 days, growing fungus was sub-cultured on PDA medium for further purification of the fungus. Furthermore, compound microscopic examinations were carried out for Mycelia and conidia structure based on the method of (Booth key 1971) to confirm that the fungus was *Fusarium oxysporum* f.sp. *lycopersici*. Standard books and research papers were also consulted during the examination of this fungus (Aneja 2004; Rifai 1969; Barnet and Hunter 1999). The purified isolates were maintained on PDA medium for further studies.

### Preparations

#### Preparation of *Fusarium oxysporum* inoculum

Using a cork-borer (1 cm), agar plugs were taken from the actively growing region of the mycelial growth for sub-culturing in other sterilized Petri dishes containing PDA medium. These were then left for seven days under fluorescent light at the room temperature. From these plates pure cultures of *Fusarium oxysporum* f.sp. *lycopersici* isolates were used for the experiment (Rampersad 2005).

#### Aqueous extract preparation

Sweet basil and River red gum leaves were collected from the Shambat area, whereas peppermint was obtained from the Omdurman Market. All samples were brought to the laboratory where they were shade dried. The samples were freed from foreign materials like stones, sand, and dust, before being kept in the laboratory for further investigation. The leaves were then milled into fine powder after previously washed with water and dried. The powdered samples were then weighted separately (25, 50 and 100 g) and placed in 75, 50 and 100 mL of sterilized distilled water respectively and placed on a shaker for 24 hrs. The extracts were then filtered overnight to obtain the concentrations 100%, 50%, and 25%.

#### Preparation of Revus Top<sup>®</sup> fungicides

The chemical tested was Revus Top<sup>®</sup> fungicide. Two mL was dissolved in 100 mL of sterilized distilled water and the final concentration of 25, 50 and 100 ppm was obtained by serial dilution test.

### Inhibition of *Fusarium* growth

Inhibition zone technique was used in this study (Rao and Srivastava 1994). The PDA medium was amended with the required concentration from sweet basil, River red gum, peppermint, and fungicide Revus Top<sup>®</sup> before being solidified in a conical flask of 250 mL containing 100 mL of PDA medium, agitated, and poured 25 mL into a

sterilized Petri dish. Three plates were assigned for each concentration and left to solidify. The other three plates with PDA medium were left to serve as a control.

Each solidified medium was then inoculated centrally by a fungal growth disc cut by a sterile cork-borer (5 mm) from an edge of an actively growing culture of the fungus *Fusarium oxysporum* f.sp. *lycopersici* grown on PDA as described above. The inoculated Petri dishes were then incubated at room temperature, with the radial growth being measured every two days. All treatments were done in triplicates and were arranged in a Complete Randomized Design.

### Calculation

The diameter of fungal radial growth was measured for every 48 hours by taking the average of two crossed dimensions for each disc in the Petri dish. The radial growth was then calculated as a percentage from the diameter (9.0 cm) of the glass Petri dish. The effect of each extract concentration on linear fungal growth was calculated as a percentage of inhibition in diameter of fungal growth:

$$\% \text{ inhibition} = \frac{dc-dt}{dc} \times 100$$

Where:

dc = Average increase in mycelial growth in control

dt = Average increase in mycelial growth in treatment

### Statistical analyses

The obtained data were statistically analyzed according to the analysis of variance (ANOVA);-Duncan's Multiple Range Test was used for mean separation using SAS software for Windows Version 9.

## RESULTS AND DISCUSSION

This study was conducted under laboratory conditions at Plant Protection Department, College of Agricultural Studies "Shambat", Sudan University of Science and Technology within the period January to March 2015. The study aimed to evaluate the antifungal activity of sweet basil, peppermint and River red gum leaves aqueous extracts and efficacy of fungicide, Revus Top®, against *Fusarium oxysporum* f.sp. *lycopersici*.

### Effect of different concentrations two days after inoculation

The results (Table 2) showed that the leaves aqueous extracts of all plants tested, and fungicide exhibited an inhibitory effect on the fungal growth after 2 days from inoculation. The percentage inhibition ranged from 5.9% at 25% concentration of River Red Gum, to 100% inhibition achieved by 50 and 100% concentrations of fungicide. Furthermore, the percentages of fungal growth inhibition was significantly high compared to the control.

Among plant extracts, sweet basil aqueous extract at all concentrations (25, 50, and 100%) gave the highest inhibition of mycelial growth (31.9, 39.9 and 51.7%). This

was followed by River red gum which gave reduction in linear growth of the fungus as (5.9, 41.3 and 43.5%) at the three concentrations (25, 50, and 100% respectively). The lowest reduction (15.5, 24.6 and 27.4%) was obtained by peppermint at the three concentrations (Table 2). Moreover, the fungicide especially at 50 and 100% concentration demonstrated 100% inhibition. However, the suppressing effect of fungicide was more pronounced (83.2, 100 and 100%) at all concentrations tested than other treatments.

### Effect of different concentrations four days after inoculation

In day four after inoculation all plant extract concentrations, as well as that of the fungicide, were invariably exhibiting suppressing effects against the fungal growth. However, all concentrations of the fungicide (25, 50, and 100%) demonstrated the significantly highest inhibition zones percent (86.1, 92.5 and 99.3% respectively). This was followed by sweet basil which gave 22.7, 42.8 and 48.5 and the lowest inhibition zone percent was given by Eucalyptus at 25 and 50% concentrations (22.4 and 39.3) . Moreover the inhibitory effect from all concentrations tested was significantly different from the control (Table 3).

### Effect of leaves aqueous extracts six days after inoculation

After six days from inoculation, the results (Table 4) showed that extracts of all the plants tested as well as the fungicide proved to be effective in suppressing the fungal growth. In fact, all tested concentrations of peppermint, sweet basil, River red gum and fungicide continued inducing significant inhibition zone percentages against test fungus compared to control (Table 4). Meanwhile, the River red gum aqueous extract at all concentrations tested (25, 50, and 100%) gave relatively more inhibitory effect (44.1, 53.1 and 53.1%) than sweet basil (36.8, 51.5 and 54.4%) and peppermint aqueous extract as well (35.5, 39.6 and 39.6%). Obviously, the test organism differs in its response to the different concentrations but on the whole, growth inhibition increased with increasing concentration. This inhibitory effect from all concentrations was significantly different from control.

### Effect of leaves aqueous extracts eight days after inoculation

After eight days from inoculation, the results (Table 5) showed that extracts of all the plants tested as well as the fungicide maintained their suppressing effect on the fungal growth. This suppressing effect of all tested concentrations of peppermint, sweet basil, River red gum and fungicide was significantly higher than the control (Table 5). However, among all treatments, the inhibitory effect of the fungicide at all concentrations was more pronouncing than others. Moreover, the assessment of the fungicide effect on fungal growth after eight days from inoculation showed a concentration dependant differential inhibition (Table 5) where the percentage inhibition increased with increasing concentration.

## Discussion

Tomato (*Lycopersicon esculentum* Mill.) is considered an important and popular plant in many countries. The global production of tomatoes doubled three times in the last 4 decades (FAO 2006). This is because of its acceptable flavor, nutritive value and ability to fruit in a wide range of environments and the relative ease with which it can be cultivated (Suarez et al. 2007). Many diseases affect tomatoes during the growing season, both in the greenhouse and field. Among these are *Fusarium* wilt disease, caused by pathogenic formae speciales of the soil-inhabiting fungus; *Fusarium oxysporum* f.sp. *lycopersici*. In fact, wilt of tomato is one of the most economically

important diseases world-wide (Rick 1979; Cal et al. 2004; Srinon et al. 2006). This pathogenic fungus remains to be a challenging task in terms of management (Rick 1976; Agrios 2005; Srinon et al. 2006).

In Sudan, tomato is becoming increasingly important for local consumption and for export. It is cultivated throughout the year under irrigation in an area that exceeds 36540 hectares, with an average yield of tons per hectare (AOAD 2007; Dawelbeit et al. 2010). Likewise, in Sudan, several diseases are known to limit production of tomato, of which *Fusarium* wilt caused by (*Fusarium oxysporum* f.sp. *lycopersici*) is one of the most important (Bhatia et al. 2004).

**Table 2.** Effect of different concentrations of plants leaf aqueous extracts and fungicide on the linear growth of *Fusarium oxysporum* f.sp. *lycopersici* in vitro two days after inoculation.

Plant extract	Products Concentrations	Inhibition zone (%)			Mean
		R1	R2	R3	
Peppermint	25%	28.5 (5.4)	10.3 (3.3)	7.6 (2.8)	15.5 (3.8)ef
	50%	14.2 (3.8)	17.2 (4.2)	42.3 (6.5)	24.6 (4.8)de
	100%	46 (6.8)	24.1 (5)	11.5 (3.5)	27.4 (5.1)de
Sweet basil	25%	28.5 (5.4)	17.2 (4.2)	50 (7.1)	31.9 (5.6)de
	50%	57.1 (7.6)	24.1 (5)	38.4 (6.2)	39.9 (6.3)cd
	100%	39.2 (7.6)	62 (7.9)	53.8 (7.4)	51.7 (7.6)bc
River red gum	25%	0 (0.07)	13.7 (3.8)	3.8 (2.1)	5.9 (2.2)fg
	50%	39.2 (6.3)	34.4 (5.9)	50 (7.1)	41.3 (6.4)cd
	100%	42.8 (6.6)	41.3 (6.5)	46.1 (6.8)	43.5 (6.7)cd
Fungicide	25%	92.8 (9.7)	75.8 (8.7)	80.7 (9)	83.2 (9.1)ab
	50%	100 (10)	100 (10)	100 (10)	100 (10.0)a
	100%	100 (10)	100 (10)	100 (10)	100 (10.0)a
Control		0 (0.7)	0 (0.7)	0 (0.7)	0.0 (0.7)g
C.V. (%)					17.03
SE±					0.46
LSD					1.725

Note: Means followed by the same letter are not significant different according to Duncan's multiple range ( $P < 0.05$ ). Data in parentheses transformed using square root transformation ( $\sqrt{X + 0.5}$ ) before analysis.

**Table 3.** Effect of leaf aqueous extracts of sweet basil, peppermint, river red gum and fungicide Revus Top® on the linear growth of *Fusarium oxysporum* f.sp. *lycopersici* in vitro four days after inoculation.

Plant extract	Treatment Concentration	Inhibition zone (%)			Mean
		R1	R2	R3	
Peppermint	25%	38.3 (6.2)	24.4 (5)	61.5 (7.9)	41.5 (6.4)bc
	50%	45 (6.7)	42.8 (6.6)	36.5 (6.1)	41.5 (6.5)bc
	100%	53.3 (7.3)	20.4 (4.6)	51.9 (7.2)	41.9 (6.4)bc
Sweet basil	25%	33.3 (5.3)	2 (1.6)	32.6 (5.8)	22.7 (4.2)c
	50%	53.3 (7.3)	32.6 (5.8)	42.3 (6.5)	42.8 (6.5)bc
	100%	55 (7.4)	34.6 (5.9)	55.7 (7.5)	48.5 (6.9)b
River red gum	25%	38.3 (6.2)	2 (1.6)	26.9 (5.2)	22.4 (4.3)c
	50%	40 (6.4)	20.4 (4.6)	50 (7.1)	36.8 (6)bc
	100%	56.6 (7.6)	28.5 (5.4)	32.6 (5.8)	39.3 (6.2)bc
Fungicide	25%	90 (9.5)	83.6 (9.2)	84.6 (9.2)	86.1 (a)
	50%	93.3 (9.7)	91.8 (9.6)	92.3 (9.6)	92.5 (a)
	100%	100 (10)	97.9 (9.9)	100 (10)	99.3 (a)
Control		0 (0.7)	0 (0.7)	0 (0.7)	0 (0.7) d
C.V. (%)					19.71
SE±					0.42
LSD					2.120

Note: Means followed by the same letter are not significant different according to Duncan's multiple range ( $P < 0.05$ ). Data in parentheses transformed using square root transformation ( $\sqrt{X + 0.5}$ ) before analysis.

**Table 4.** Effect of leaf aqueous extracts of sweet basil, peppermint, River red gum and fungicide Revus Top on the linear growth of *Fusarium oxysporum* f.sp. *lycopersici* in vitro six days after inoculation.

Treatments	Inhibition zone (%)			Mean		
	Plant extract	Concentrations	R1		R2	R3
Peppermint		25%	40.3 (6.4)	16.6 (4.1)	49.5 (7.1)	35.5 (5.8) d
		50%	53.8 (7.4)	34.3 (5.9)	30.6 (5.6)	39.6 (6.3) bcd
		100%	25.9 (5.1)	35.2 (6)	58.4 (7.7)	39.9 (6.2) bcd
Sweet basil		25%	41.3 (6.5)	35.2 (6)	33.6 (5.8)	36.8 (6.1) cd
		50%	51.9 (7.2)	48 (7)	54.4 (7.4)	51.5 (7.2) bc
		100%	55.7 (7.5)	54.9 (7.4)	53.4 (7.3)	54.7 (7.4) b
River red gum		25%	49 (7)	43.1 (6.6)	40.5 (6.4)	44.3 (6.6) bcd
		50%	55.7 (7.5)	50.9 (7.2)	52.4 (7.3)	53.1 (7.3) bc
		100%	55.7 (7.5)	51.9 (7.2)	51.4 (7.2)	53.1 (7.3) bc
Fungicide		25%	94.2 (9.7)	92.1 (9.6)	92 (9.6)	92.8 (9.6) a
		50%	92.3 (9.6)	96 (9.8)	92 (9.6)	93.5 (9.6) a
		100%	99 (10)	100 (10)	100 (10)	99.7 (10) a
Control			0 (0.7)	0 (0.7)	0 (0.7)	0 (0.7)e
C.V. (%)						13.04
SE±						0.40
LSD						1.090

Note: Means followed by the same letter are not significantly different according to Duncan's multiple range ( $P < 0.05$ ). Data in parentheses transformed using square root transformation ( $\sqrt{X + 0.5}$ ) before analysis

**Table 5.** Effect of different concentrations of plant leaf aqueous extracts and fungicide on the linear growth of *Fusarium oxysporum* f.sp. *lycopersici* in vitro eight days after inoculation.

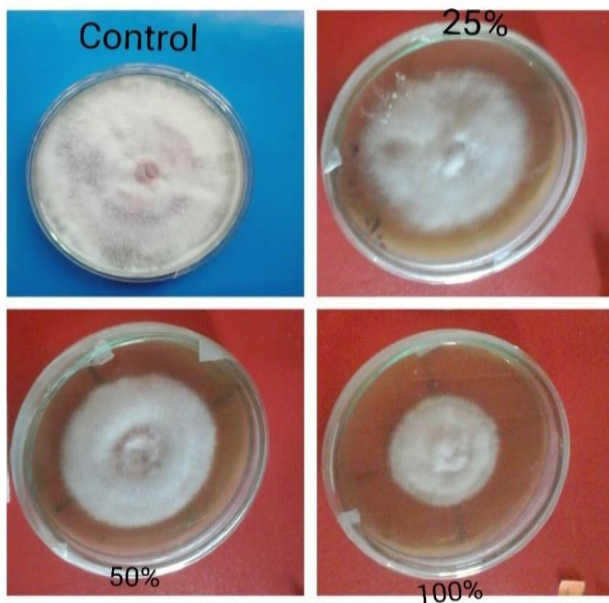
Treatments	Inhibition zone (%)			Mean		
	Plant extract	Concentrations	R1		R2	R3
Peppermint		25%	38.2 (6.2)	19.4 (4.5)	53.3 (7.3)	37.0 (6) c
		50%	21.9 (4.7)	38.8 (6.3)	51.8 (7.2)	37.5 (6) c
		100%	58.5 (7.7)	49.2 (7.1)	40.7 (6.4)	49.5 (7) bc
Sweet basil		25%	39.8 (6.4)	40.2 (6.4)	41.4 (6.5)	40.5 (6.4) c
		50%	49.5 (7.1)	46.2 (6.8)	46.6 (6.9)	47.5 (6.9) bc
		100%	54.4 (7.4)	58.2 (7.7)	57 (7.6)	56.6 (7.5) b
River red gum		25%	38.2 (6.2)	41 (6.4)	40.7 (6.4)	40.0 (6.2) c
		50%	36.5 (6.1)	42.5 (6.6)	41.4 (6.5)	40.2 (6.4) c
		100%	43 (6.6)	41 (6.4)	45.1 (6.8)	43.1 (6.6) bc
Fungicide		25%	95.1 (9.8)	94 (9.7)	94 (9.7)	94.4 (9.7) a
		50%	93.4 (9.7)	97 (9.9)	94 (9.7)	94.9 (9.7) a
		100%	99.1 (10)	100 (10)	100 (10)	99.7 (10) a
Control			0 (0.7)	0 (0.7)	0 (0.7)	0 (0.7)d
C.V. (%)						8.25
SE±						0.38
LSD						0.9553

Note: Means followed by the same letter are not significant different according to Duncan's multiple range ( $P < 0.05$ ). Data in parentheses transformed using square root transformation ( $\sqrt{X + 0.5}$ ) before analysis

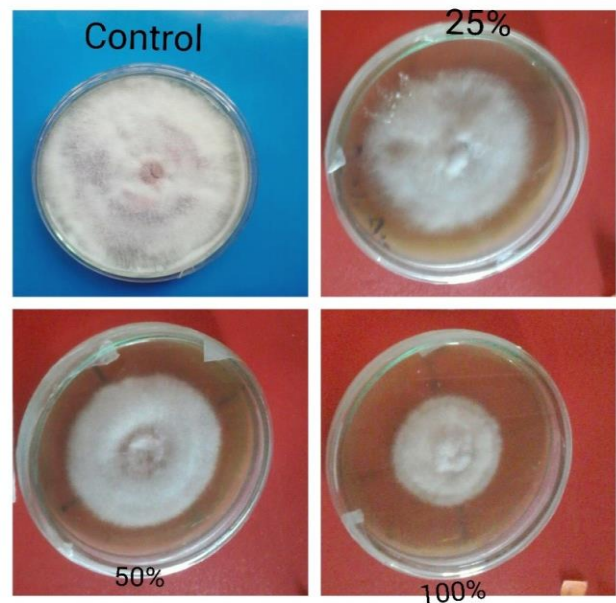
Several research findings have presented strategies to control this fungal pathogen (Haware and Nene 1982; Jiménez-Díaz et al. 1993; Biondi et al. 2004; Ahmed 2011). However, management of seed-borne and soil-borne diseases such as tomato wilt caused by *Fusarium oxysporum* f.sp. *lycopersici* has always been problematic (Rao and Balachadran 2002). Generally, the use of synthetic fungicides considerably reduced wilt incidence in tomato, but their use is costly as well as environmentally undesirable (Song and Goodman 2001). Moreover, the use of resistant varieties is faced with the breakdown of resistance due to high pathogenic variability in the pathogen population (Kutama et al. 2011; 2013). In this

context, the search for an eco-friendly way of managing *Fusarium* wilt in tomato which offers an alternative to fungicides is highly demanding.

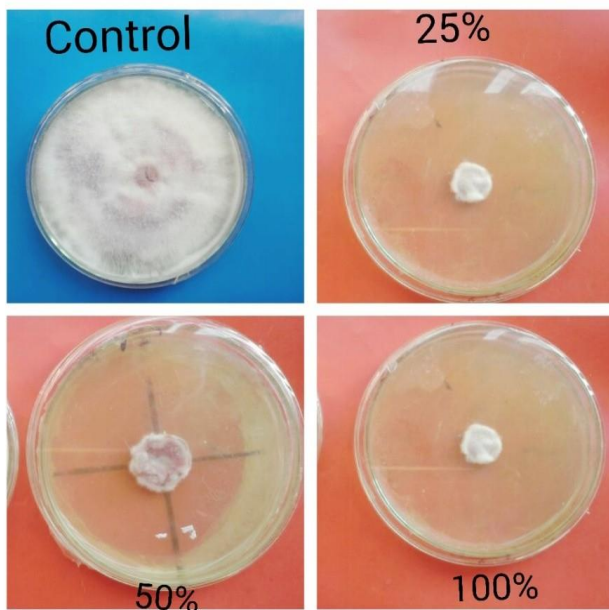
Fortunately, progress achieved in recognizing antimicrobial compounds in plants gave more promises in combating plant pathogenic diseases. Such compounds, being biodegradable and selective in their toxicity, are considered valuable for controlling some plant diseases (Schmutterer 2002). In fact, plants with biologically active secondary metabolites are extremely abundant, where over 80% of all known Alkaloids, Terpenoid, Phenols and other secondary metabolite were produced from them (Siddiq 1993; Newman et al. 2000).



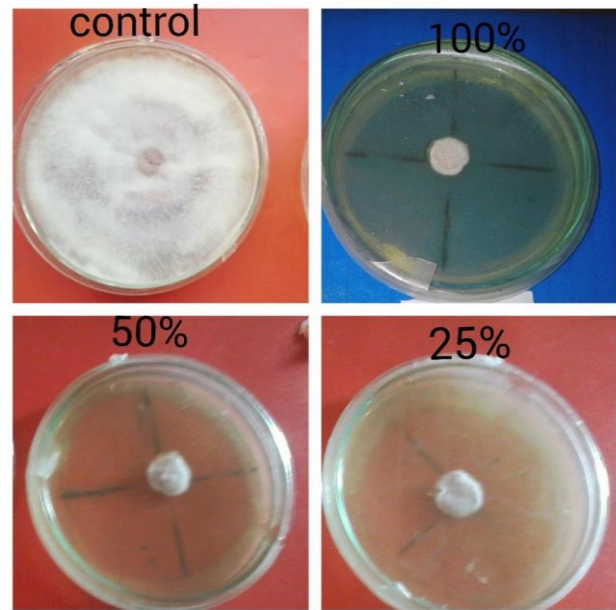
**Figure 1.** Effect of leaf aqueous extracts of peppermint on growth of *Fusarium oxysporum* f.sp. *lycopersici* in vitro.



**Figure 3.** Effect of leaf aqueous extracts river red gum on the growth of *Fusarium oxysporum* f.sp. *lycopersici* in vitro.



**Figure 2.** Effect of leaf aqueous extracts of sweet basil on the growth of *Fusarium oxysporum* f.sp. *lycopersici* in vitro.



**Figure 4.** Effect of leaf aqueous extracts fungicide (Revus Top<sup>®</sup>) on the growth of *Fusarium oxysporum* f.sp. *lycopersici* in vitro.

The results (Tables 2-5) revealed that the sweet basil, peppermint, and River red gum leaves aqueous extracts and fungicide, Revus Top<sup>®</sup>, solution consistently and throughout the course of the experiments exhibited an inhibitory effect on the mycelial radial growth of the fungus with significantly higher inhibition reduction growth percent compared to control. Similar studies which explored the effect of extracts of many higher plants and their essential oils have been reported to exhibit antibacterial, antifungal, and insecticidal properties under

laboratory trials (Agrafotis 2002; Ergene et. al. 2006; Kiran and Raveesha 2006; Okigbo and Ogbonnaya 2006; Shariff et. al. 2006). In fact, this finding agrees with Muntasir (2014) who tested the bioactivity of sweet basil extract against fungi and demonstrated its suppressing effect on the fungal growth in vitro. Bansal and Rajesh (2000) also reported the antifungal effect of River red gum.

As demonstrated by various research, there is considerable interest in the use of peppermint for controlling various fungal diseases in plants (Kalemba

2003; Soković et al. 2009). Similar results were found in Moghtader (2013), who tested the effect of essential oil of its *Mentha piperita* L. and its comparison with synthetic menthol on *Aspergillus niger*. These well-known Bicarbonates are widely used in the food industry and were found to suppress several fungal diseases of cucumber plants.

The data presented in this study showed that the use of sweet basil in vitro expressed an inhibitory effect against the mycelial growth of *Fusarium oxysporum*, and the percentage zone of inhibition was significantly higher than the control. The obtained results were in line with that of Katooli et al. (2012) who tested the antifungal activity of River red gum (*Eucalyptus camaldulensis*) essential oil evaluated on suppressed the mycelial growth of postharvest pathogenic fungi, *Penicillium digitatum*, *Aspergillus flavus*, *Colletotrichum gloeosporioides* and soilborne pathogenic fungi, *Pythium ultimum*, *Rhizoctonia solani*, and *Bipolaris sorokiniana* pathogenic fungi.

Generally, uses of synthetic fungicides considerably reduces the impact of this disease. In this study, the fungicide Revus Top® consistently inhibited the radial mycelial growth of *Natrassia mangiferae* and its suppressing effect was pronounced at all concentrations tested throughout the time of the investigation. These results confirm that which were reported by Themis et al. (2005), who indicated the effectiveness of fungicides against other fungi that infect limb dieback of figs in California.

The leaf aqueous extracts of all plants tested exhibited an inhibitory effect on fungal growth. Thus, the two components plus fungicide (Revus Top®) could be applied as part of an integrated approach to control *Fusarium* wilt in tomato.

The sweet basil plant leaf aqueous extract exhibited a more inhibitory effect than that of the river red gum and peppermint. This finding is the first one of its kind in Sudan in the in vitro of *Fusarium* wilt control in tomato, which suggests more investigation is needed.

The screened concentrations of river red gum, sweet basil and peppermint leaves aqueous extracts differ in their reactions to test fungus. Likewise, the test organism responded differently to the different concentrations of extracts. This variability in response which expressed by test organism may be used to adjust an optimum dose for controlling *Fusarium* wilt in tomato.

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## Short Communication: Influence of the content level of teichoic acids in the cells of *Staphylococcus aureus* on their adhesive properties

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**Abstract.** Minukhin VV, Kochneva EV. 2017. Short Communication: Influence of the content level of teichoic acids in the cells of *Staphylococcus aureus* on their adhesive properties. *Bioteknologi* 14: 9-11. Teichoic acids (TA) are known to be factors of pathogenicity and virulence. They take part in binding the bacteria *Staphylococcus aureus* to epithelial cells of mucous membranes. The purpose of the research consisted in determining the content of TA in cells of clinical and reference strains of *S.aureus* and studying the relationship between these acids and adhesive properties of the cell wall. TA from cells of *S.aureus* were extracted by the addition of 10 % trichloroacetic acid to washings of agar cultures; followed by precipitation with cold ethanol, washing with acetone, ethanol, and ether in a desiccator. The content of TA was determined at a SP-46 by optic density (OD) with wavelength  $\lambda = 254$  nm. When TA were isolated from cells of clinical and reference strains of *S.aureus*, it was revealed that their content levels differed. Values of the clinical strains were higher than those of the reference ones: respectively,  $(0.373 \pm 0.016)$  versus  $(0.147 \pm 0.014)$  OD units. This phenomenon could be explained by a high degree of aggressiveness of the clinical strains and could influence the activation of the complement that in its turn caused a systemic response resulting in a decrease of phagocytic activity. The course of the research revealed a direct correlation dependence of the content of TA upon adhesion values ( $r = 0.643$ ). This dependence could affect the ability of microorganisms to form biofilms.

**Keywords:** Adhesion, *Staphylococcus aureus*, teichoic acids

### INTRODUCTION

Teichoic acids (TA) compose a significant part of the cell wall in gram-positive bacteria and their proportion can reach to 60 % of its weight (Archibald 1993; Baddiley et al. 1961). These acids have covalent links with peptidoglycans and are an integral part of the cell wall. Thus, they are in close contact with all the processes that take place with its participation. These include growth and division of cells, binding and redundancy of cations required for the functioning of membrane enzymes, processes of intercellular recognition, reception of phages, pathogenicity. TA and other anionic compounds of the cell wall make a significant contribution to the formation of polyelectrolyte gel structure and determine its mechanical properties (Archibald et al. 1961; Archibald 1988).

The study of the cell walls of pathogenic microorganisms has led to the understanding of such important phenomena as adhesion, virulence, and formation of biofilms on implanted materials (Cramton et al. 1999; Xia 2010). Side by side with other components of cell walls, TA are responsible for sensitivity of bacteria to some antibiotics and their immunomodulatory properties (Sieradzki 1999; Stephanie et al. 2012). Some anionic polymers of cell walls of bacteria participate in processes of intercellular adhesion, which are based on ionic interactions of adhesin proteins of one cell with specific receptors of another one. TA can be such receptors (Archibald 1974; Sanderson 1962).

According to published literature, TA of *Staphylococcus aureus* take part in binding bacteria to epithelial cells of mucous membranes (Aly et al. 1980; Naumova et al. 2001; Scheffers 2005). They launch a complementary cascade along an alternative route, activate the clotting and kallikrein-kinin systems. *Staphylococcus aureus* is a common cause of infections associated with colonization of implanted biomaterials. The molecular basis of the initial adhesion of bacteria to artificial surfaces has not been fully clarified. But it is known that the key part of the first stage of biofilm formation is played by TA. The processes of interaction between TA and biomaterials are influenced by ionic forces and those of van der Waals, which can be both attractive and repulsive (Bera 2007; Matthias et al. 2001).

The negative charge of TA play a crucial part in the coaggregation of cations, binding autolysins as well as in pathogenic and adhesive properties, caused by the phosphate groups. Meanwhile, the role of certain structural elements of these polymers has not been studied enough, though it has been revealed that they affect the functional activity and some biological properties of TA (Biswas et al. 2012; Matthias et al. 2001).

Thus, the adhesive and virulent properties of bacteria, their ability to interact with different positively charged molecules, polymers, etc., depend upon the degree of esterification of TA. Hence one of the stages of this research consisted in revealing the relationship between adhesive properties and content of TA in the cell wall of *S.aureus*.

The objective of this research was to determine the content of TA in cells of clinical and reference strains of *S. aureus* and study the relationship between these acids and adhesive properties of the cell wall.

## MATERIALS AND METHODS

The research used 55 clinical strains isolated from patients with different proinflammatory infections and four reference strains (ATCC 25923) as the control group. The strains were then divided into four groups. The first group consisted of strains isolated from a purulent discharge of wounds in acute inflammatory processes (abscess, gangrene, acute purulent periostitis, phlegmon, acute appendicitis, acute hidradenitis, acute mastitis). The second group of strains were isolated from a purulent discharge of wounds in protracted inflammatory processes (furunculosis, carbuncle, infected wounds, panaritium, atheroma, hematoma, erysipelas, baroclinities). The third group of strains were isolated from mucus of the fauces and nose during an examination for carriage. The remaining reference strains ATCC 25923 were the control group.

Pure cultures were isolated in compliance with standard methods (MacFaddin 2000; David et al. 2004; Murray et al. 2011).

TA from cells of *S. aureus* were extracted by addition of 10 % trichloroacetic acid to washings of agar cultures followed by precipitation with cold ethanol, washing with acetone, ethanol, and ether in a desiccator. The content of TA was determined at an SP-46 by optic density (OD) with wavelength  $\lambda = 254$  nm.

## RESULTS AND DISCUSSION

The results of this study showed that the values of TA content in *S. aureus* in clinical and reference strains differed reliably ( $p < 0.05$ ) (Table 1).

The values of average optical density were ( $0.542 \pm 0.016$ ) OD units in the 1<sup>st</sup> group and ( $0.147 \pm 0.014$ ) in the 4<sup>th</sup> one, thereby making a statistical difference. The values in the 2<sup>nd</sup> and 3<sup>rd</sup> groups were less versus the 1<sup>st</sup> one, but higher than in the reference strains. The values for these groups were, respectively, ( $0.381 \pm 0.019$ ) and ( $0.196 \pm 0.013$ ) OD units.

Thus, it was revealed that the content of TA in cells of *S. aureus* was higher in clinical isolates versus the reference strains, which might be caused by the degree of their aggressiveness and influenced the course of an infectious disease.

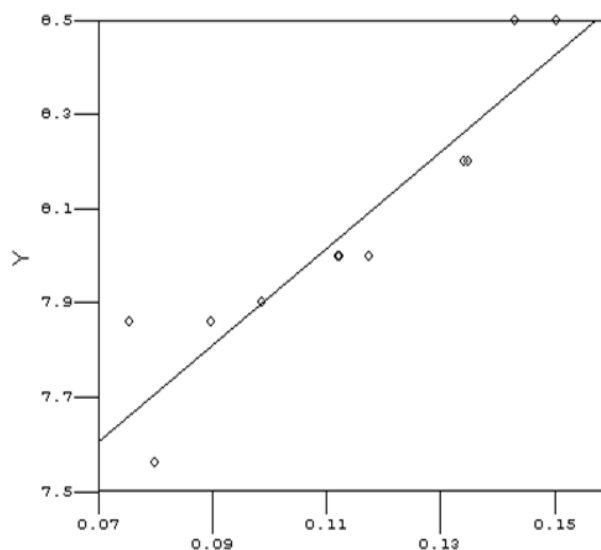
Taking into consideration that the content of TA is closely associated with adhesive properties of *S. aureus*, this research revealed a correlation between these indices (Figure 1).

The study of the relation between adhesive property values and TA content in cells of *S. aureus* revealed a direct correlation dependence ( $r = 0.643$ ), which reduced the ability of microorganisms to form microfilms.

**Table 1.** Mean values of TA content in cells of *S. aureus*

Groups of <i>S. aureus</i> strains studied	TA content (OD units)
Acute inflammatory processes (abscess, gangrene, acute purulent periostitis, etc.), n = 25	$0.542 \pm 0.016^*$
Protracted inflammatory processes (furunculosis, carbuncle, etc.), n = 25	$0.381 \pm 0.019^*$
Examination of carriers, n = 5	$0.196 \pm 0.013^*$
Reference strains ATCC 25923, n = 4	$0.147 \pm 0.014^*$

Note: \* – the difference is reliable,  $p < 0.05$ ; results of 3 repeated studies are presented



n = 11  
Regression coefficient: deviation b – 0.1112, shift a – “+”3.8;  
Correlation coefficient:  $r = 0.643$ ,  $t = 10.62$ .

**Figure 1.** Analysis of the correlation dependence of the content of TA upon adhesive properties of *S. aureus* strains (a direct stable correlation dependence).

In conclusion, the results of this study demonstrated that the content of TA in cells of *S. aureus* was higher ( $p < 0.05$ ) in clinical isolates versus the reference ones: average OD was, respectively, ( $0.373 \pm 0.016$ ) and ( $0.147 \pm 0.014$ ) OD units. This phenomenon could be explained by a higher degree of aggressiveness of the clinical strains and could influence the activation of the complement that in its turn caused a systemic response resulting in a decrease of the phagocytic activity. A direct correlation dependence of the content of TA upon adhesion values ( $r = 0.643$ ) was found out. This dependence could affect the ability of microorganisms to form biofilms. Study of the ability of *S. aureus* strains to form biofilms and revealing of the correlation of the above formation with the content of TA are promising directions for further research.

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## Short Communication: Identification of structural and elemental composition of *Cassia obtusifolia*

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**Abstract.** Ahmed AYA, Marouf AAS. 2017. Short Communication: Identification of structural and elemental composition of *Cassia obtusifolia*. *Bioteknologi 14*: 12-15. Kawal is a dried fermentation product of sicklepod (*Cassia obtusifolia*) leaves. The people of Sudan and Chad use kawal as a meat substitute. In this study, kawal was obtained in powder form from Forbranga, Darfour. The objective of this study was identification the chemical compositions of kawal. FTIR spectrum and atomic absorption spectrophotometer methods were used. In FTIR experimental procedure, the sample was used as a dried powder. While for the atomic absorption and inductively coupled plasma, the sample was used after combustion for extracting the minerals (K, P, Na, Mg, Ca, Zn and Fe). The investigations with FTIR showed that the fermented leaves of *C. obtusifolia* (Kawal) consisted of several types of molecular functional groups: alkyl halide, alkene, a nitro functional group, aromatic, carbonyl, alcohol, esters, ether, and acid and amides. Mineral contents of the samples were identified. About 2.50% is the presence of calcium, with the other minerals as Mg 0.66%, P 0.393%, Na 0.165% and K about 1.595%. Moreover, for extracting the iron (Fe) and zinc (Zn) minerals of fermented cassia leaves, the atomic absorption spectrophotometer was used and found that Zn was 0.665mg/L and Fe consist of the value 15.6411mg/L.

**Keywords:** *Cassia obtusifolia*, fermented leaves, spectroscopic

### INTRODUCTION

*Cassia obtusifolia* (Leguminosae), also known as sicklepod, is a plant that grows wild in Africa. It is found in wastelands in the rainy season. The people from western Sudan and eastern Chad benefit from the plant, due to its leaves can be fermented to become protein-rich food. This food is known as kawal. Considered as an alternative source of meat or meat extender, kawal has had a significant role in providing protein during famine years. It has probably protected many children against kwashiorkor. However, because of its pungent, fetid odor that lingers on the fingers for hours, kawal has been refused by the elite who consider it unfit for modern social life (Algadi and Yousif 2015).

Study of the nutritional quality of *C. obtusifolia* leaves and kawal (their chemical composition, protein fractions, in vitro protein digestibility, amino acid contents, mineral content, phytic acid, tannin and total polyphenol of cassia leaves) revealed that fermentation in the kawal processing has resulted in the increase of in vitro protein digestibility, fat, protein and ash content and a decrease in fiber content. (Algadi and Yousif, 2015; Algadi, 2008). Moreover, previous studies by Nuha et al. (2010) also indicated the changes in the chemical composition, anti-nutrients, and extractable minerals among the green leaves, kawal and cooked leaves of *Cassia*. Their samples were as a dry powder. They found that the protein and ash contents increased after cooked, the total energy of both samples were decreased after fermentation process and cooked. Furthermore, anti-nutritional factors (tannin, phytate, total polyphenols) also

decreased in the treated samples, but there is an increase in the total major minerals. Total polyphenols by using spectrophotometer method by Price and Bulter (1977), minerals composition by Chapman and Pratt (1982) and HCl extractability by using Chauhan and Mahjan (1988).

Mbaiguinam et al. (2005) studied the chemical components of kawal and found aliphatic acids, and they identified as hexanoic acid (27%), butyric acid (10.4%), and valeric acid (6.3%) by using laser amounts of p-ethylphenol and p-methyl phenol. They also found a higher portion of protein fraction of the crude leaves (20.2%), compared to fermented leaves (kawal) (12.9%). They used dried samples for gas chromatography and gas chromatography-mass spectrometry (GC/MS), to analyze volatile compounds. For amino acid analysis, the kawal and leaves were hydrolyzed under nitrogen and HCl vapor by using picotag work, followed by chromatography for Amino acid identification. The objectives of this study were spectroscopic studies of the dry fermented *C. obtusifolia* leaves.

### MATERIALS AND METHODS

#### Materials

The fermented leaves of *Cassia obtusifolia*, or kawal, were obtained from Forbranga (western Sudan). They were obtained in dry form. After being sun-dried and freed from foreign materials, they were powdered by hummer mill with the same mesh size. They were then kept in clean bottles at room temperature for further use (Figure 1).



**Figure 1.** Sun-dried powder of kawal (*Cassia obtusifolia*).

### Methods

Samples were analyzed at room temperature. To identify the presence of certain functional groups in the sample, IR spectrum was recorded on Fourier transformer spectrometer FTIR-8400 spectrophotometer (Shimadzu, Kyoto, Japan). For further analysis, the atomic absorption spectrophotometer (model AA-6800, Shimadzu) was used to identify the mineral content (Ca, Na, K, Mg, P, Zn and Fe) composition of the sample.

#### FTIR analysis

In this experiment, 100mg from potassium bromide (KBr) was used additionally to 1 mg sample of the dry powder kawal. Furthermore, KBR and the sample were mixed until it became a mixture. The mixture samples were compressed and afterwards put on the piston. They were then applied to the Fourier transformer spectrometer device for detection of mineral contents of fermented cassia leaves.

#### Digestion method

Ca, Na, K, Mg and P digestion method was applied to prepare the sample for spectroscopy analysis. The sample was digested in  $\text{HNO}_3/\text{HCl}_4$ , and elements were detected by inductively coupled plasma spectroscopy (ICPS). Heater, test tube, and flasks were used as apparatus. 2g of the sample was weighed into test tubes, 8 mL  $\text{HNO}_3$  was added to the test tubes and covered them with glass ball and left it to stand for overnight. Another  $\text{HNO}_3$  produced fumes and was added as did it in the chamber, 3 mL of  $\text{HClO}_4$  acid was added and heated slowly until almost dry. Moreover, the glass ball was removed and heated strongly until white fumes disappeared. The tubes were cooled and 5 mL of 0.1N HCl was added and boiled gently for 30 minutes after leaving it to cool. The solution was then transferred into the 50 mL flask. The tubes were rinsed with 10 mL of 0.1N HCl and boiled gently for 10 minutes, the solution was

poured into the previously flask and filled it to 50 mL mark with 0.1N HCl. The ICP readings were then recorded.

#### Atomic Spectroscopic Method

For analyzing iron and zinc, the atomic spectroscopy method was used after combusting the sample. The optic parameters were set in the following specification: lamp current low (Am) (12 and 8), wavelength (nm) 248.3 and 213.9, slit width (nm) (0.2 and 0.5) and lamp mode BGC-D2. The atomizer/gas-flow was set up as follows: fuel gas flow rate (L/min) (2.2 and 2), flame type Air-C<sub>2</sub>H<sub>2</sub>, burner height (mm) (9 and 7), burner lateral pos (pulse) 0 and burner angle (degree) 0. and measurements parameters are: order first, zero intercept no, unit mg/L, repetition sequence SM-M-M-..., pre-spray time (sec) 3, integration time (sec) 5 and response time 1.

## RESULTS AND DISCUSSIONS

FTIR spectrum of fermented *Cassia obtusifolia* leaves revealed several regions of frequencies that can lead to identifying of molecule group. Chemical compositions of fermented kawal are shown in Figure 2. Kawal was found to consist of several types of molecules, with three functional groups. On the alkyl-halide functional group was found C-Br which has strong intensity vibration stretch, C-Cl with strong intensity of stretching moves, and C-F molecular with the same prescriptions. While the alkene functional group consist of C-H with bending strong mode of vibration, the two-C-H alkenes have bending mode of vibration. However, their intensity is variable as the three C=C alkenes have variable intensity with stretch motion and the two C-H with the strong stretch mode of vibration.

The absorption frequencies also identified N-O which belong to the nitro functional group with stretch vibration mode and strong intensity. The C=C is an aromatic molecule, stretch mode and its intensity is medium weak or multiple bands. C=O is carbonyl with strong stretch. O-H is alcohol structure which has stretch, H-bonded vibration and strong broad intensity. The two C-O are in the ester function group, with stretch vibration mode and two bonds or more intensity. The ether C-O with the same descriptions of the ester. The acid C-O stretch strong intensity of vibration. Lastly, the two N-H amides have bending modes of vibrations and their intensity are medium (Silverstein et al. 2014).

The minerals (calcium, magnesium, phosphorus, sodium, and potassium) in kawal is reported in Table 1. Calcium and potassium have relatively high proportions compared to other minerals. About 2.50% was found as Calcium and the other minerals, magnesium 0.66%, phosphorus 0.396%, sodium 0.165% and potassium about 1.595%. These results indicat the fermented leaves of *C. obtusifolia* contain the major elements needed by the human body (Table 1).



**Table 1.** Mineral elements contents of kawal.

The element	Amount of kawal
Calcium	2.50%
Magnesium	0.66%
Phosphorus	0.396%
Sodium	0.165%
Potassium	1.595%

**Table 2.** Content of Zn and Fe of sample Mg/L.

Mineral	Content (mg/L)
Zn	0.6655
Fe	15.6411

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## Short Communication: Inhibitory effect of *Sapindus rarak* ethyl acetate extract on *Staphylococcus aureus*

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**Abstract.** *Silviani S. 2017. Short Communication: Inhibitory effect of Sapindus rarak ethyl acetate extract on Staphylococcus aureus. Bioteknologi 14:1.* The research aimed to know the Minimum Inhibitory Concentration (MIC), the Minimum Killing Concentration (MKC) and the inhibitory fungal zone of *Sapindus rarak* - ethyl acetate extract on *Staphylococcus aureus*. The research used an experimental descriptive design by using controlled post-test approach by measuring the value of MIC, MKC and the inhibitory fungal zone. This research was performed in 2016 at the Bacteriology Laboratory of Akademi Analis Kesehatan Nasional (Academy of National Health Analyst) by using dilution method and diffusion disc. The result of this research showed that the value of MIC was not able to be concluded, the value of MKC was 80% and the greatest inhibitory effect zone achieved at 100% concentration was 10.3 mm. The ethyl acetate extract claimed medium to strong inhibitory effect on *Staphylococcus aureus*.

**Keywords:** Inhibitory effect, *Staphylococcus aureus*, ethyl acetate extract, MIC, MKC

### INTRODUCTION

Indonesia is rich in natural resources which provide benefit for human health. *Sapindus rarak* (Sapindaceae), because of its chemical content like alkaloid, tannin, polyphenol, flavonoid, and saponin is believed to be a useful bio-resource with antibacterial potentialities (Silviani and Puspitaningrum 2015). The plant can be used as batik washing material and is easily available in Indonesia.

Acne is a blockage of skin and hair oil gland channel marked by skin inflammation. (Rahmi et al. 2015). Acne is familiar to society, especially to teenagers, and might cause psychological problems (Uhlenhake et al. 2010). Eighty-five percent of acne cases belong to females of age 14-17 years and males of age 16-19 years (Hasan et al. 2015).

*Staphylococcus aureus* inflicts acne by invading hair follicle tissue, multiplying and then causes necrosis (Razak et al. 2013). In some countries, the bacteria has been recognized to be resistant to penicillin group, including methicillin (Hilda et al. 2015). Khusnan et al. (2016) states that *S. aureus* is resistant to penicillin 78%, doxycycline 56%, gentamicin 26%, tetracycline 22%, erythromycin 13% and methicillin 9%. Morell and Balkin (2011) state that there is a higher resistance of *S. aureus* to antibiotic from 2% to 64% in 2004. Recently, natural bioresources are chosen by people as one of the alternatives to prevent resistance and minimize the side effect (Fatisa 2013).

Based on the above, this research was performed to explore the antibacterial properties of *S. rarak* against *S. aureus* based on the value of Minimum Inhibitory Concentration (MIC), Minimum Killing Concentration (MKC) and the diffusion disc.

### MATERIALS AND METHODS

#### Materials

Materials used in this research were the fresh fruit of *Sapindus rarak*, obtained from Sky Argo Distributor in Solo. While the *S. aureus* isolate was obtained from agents with acne. This research was performed by using experimental analytical design and controlled post-test approach. *Quota sampling* was the technique chosen.

#### Extraction preparation

Brown *S. rarak* fruit with diameters of 2 cm were chosen. The fruit were then cleaned, seeds were taken out, and then dried. Dried *S. rarak* were meshed using a 20-mesh sieve and kept in a closed container (Samsuharto 2010).

#### Making ethyl acetate extract

Ethyl Acetate Extraction was done by using maceration method. Maceration is a process of extracting simplicia by shaking or stirring with ethyl acetate solvent in room-temperature for 5 days. Then, the extract was obtained after filtration and evaporation of ethyl acetate solution until a constant weight. The ethyl acetate extract was then tested by using qualitative phytochemical test.

#### Checking the Minimum Inhibitory Concentration

1 ml nutrient broth was added into 8 test tubes. Tubes 1-6 were set for concentration sample of 75% to 100%, tube 7 was set for positive control and tube 8 was for negative control. A suspension of 1 ml bacteria, adjusted to McFarland standard no 0.5 which had been diluted 100X,

was then added to each of the tubes, number 1-8. Tubes number 1-7 were filled with *S. rarak* ethyl acetate extract with varied concentration and tube number 8 was filled with *S. rarak* ethyl acetate extract of 100% concentration. All tubes were then incubated at 37°C for 24 hours. The turbidity of each tube was noted.

### Checking the Minimum Killing Concentration

36 ose (1 mm diameter) of each tube was inoculated to a Nutrient Agar Plate media by streaking. All tubes were incubated at 37°C for 24 hours. The colony growth in every plate was noted.

### Checking Diffusion Disc

The bacterial suspension was inoculated with density  $1.0 \times 10^8$  CFU/ml into a Nutrient Agar plate evenly. Then the blank disc that had been filled with *S. rarak* ethyl acetate extract was set. All tubes were then incubated at 37°C for 24 hours (CLSI 2006).

## RESULTS AND DISCUSSION

The phytochemical test of ethyl acetate extract was shown in Table 1. Based on Table 1, it was confirmed that *S. rarak* - ethyl acetate extract has antibacterial properties as indicated by its secondary metabolites substances or active materials.

Table 2 revealed that the value of MIC could not be concluded because there was turbidity in *S. rarak* extract control, thus the turbidity in the concentration treatment might result from *S. rarak* - ethyl acetate extract.

Based on Figure 1, it is shown that increasing concentration of extracts formed a wider inhibitory effect zone. *S. aureus* is a gram-positive bacteria, coccus and grouped like grapes (Radji 2011). *S. aureus* has optimum growing temperature at 37°C, best pigment formation takes place at 20-25°C. The bacteria has a grayish to golden yellow, round-shaped, smooth, distinctive, and shiny colony (Jawetz 2008). *S. aureus* can be differed from other *Staphylococcus* based on mannitol fermentation test, coagulation and the pigment produced (Toele and Lenda 2008). Coagulation enzyme is pathogenic factor *S. aureus* (Andreasen 2008). *S. aureus* cell wall is composed of peptidoglycan which is a polysaccharide polymer. Peptidoglycan can be damaged by strong acid and lysozyme.

*Sapindus rarak* consists of active compound, tannin, saponin, polyphenol and flavonoid. The result of qualitative Test (Table 1) shows that ethyl acetate extract contains all those active compounds, it is similar to the research performed by Silviani and Puspitaningrum (2015) which states that *S. rarak* contains polyphenol, tannin, saponin and flavonoid.

The tannin present in the extract gives antibacterial to *S. aureus* by disturbing the permeability of bacteria cell walls (Retnowati et al. 2011). Payne et al (2012) reported that tannic acid in tannin inhibited *S. aureus* biofilm formation. It can also inhibit the works of DNA enzyme topoisomerase and reverse transcriptase so that induces the

death of bacteria cell (Amelia, 2015). Flavonoid is a phenol compound with a hydroxyl group, this compound has polar characteristics and works by forming an extracellular complex with bacteria protein so that it damaged bacteria cell wall (Bansode and Chavan 2012).

Alkaloid works as antibacterial substance by disturbing the forming layers of bacteria cell wall, especially on peptidoglycan site. (Paju 2013). The inhibition of the cell wall formation causes the death of bacteria. Tannin and flavonoid compounds are mostly drawn by using ethyl acetate solvent. Because ethyl acetate is a semi-polar solvent, tannin and flavonoid concentration are higher in ethyl acetate than in any other extracts (Tanaya et al. 2015).

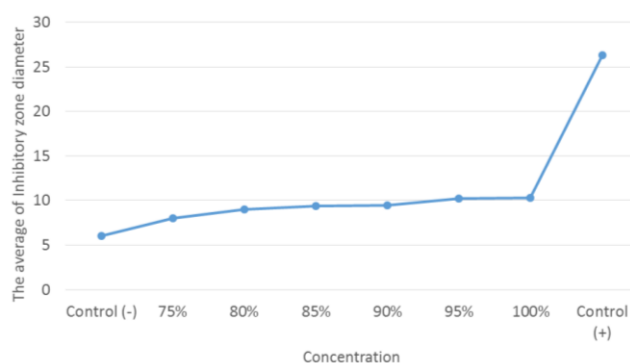
**Table 1.** Phytochemical test on ethyl acetate extract of *S. rarak*

Active material	Result	Conclusion
Flavonoid	Red-yellow color	Positive
Alkaloid	Orange sediment after the addition of HCL & dragendroff	Positive
Saponin	Forming stable height of foam 1-3 cm for 10' after shaking	Positive
Tannin	Bluish green after the addition of water and FeCl <sub>3</sub>	Positive
Polyphenol	Black-blue color after the addition of FeCl <sub>3</sub>	Positive

**Table 2.** MIC and MKC ethyl acetate extract to *Staphylococcus aureus*

Concentration	MIC	MKC
100%	+	0
95%	+	0
90%	+	0
85%	+	0
80%	+	0
75%	+	1
Sapindus rarak control	+	0
Bacteria control	+	UC
Media control	-	0

Note: Uc = Uncountable, + = Turbidity, - = Non-Turbidity. MIC = Minimum Inhibitory Concentration, MKC = Minimum Killing Concentration



**Figure 1.** Average of inhibitory zone diameter of ethyl acetate extract to *Staphylococcus aureus*

The result of *S. rarak* - ethyl acetate extract diffusion disc to *S. aureus* (Figure 1) could not be compared to control (+), that is ciprofloxacin. As previously mentioned by Davis and Stouts (1971), antibacterial inhibitory level of natural materials can be classified into 4 categories. They are: weak if the inhibitory level is  $\leq 5$  mm, medium if the inhibitory level is 5-10 mm, strong if the inhibitory level is 10-19 mm, and very strong if the inhibitory level established is more than 20 mm. In this research, the inhibitory zone of ethyl acetate extract was medium to strong. 80% extract concentration resulted medium inhibitory level, 85%-100% concentration resulted strong inhibitory level.

The results support the earlier report by Marsa's (2010) which stated that *S. rarak* extract was able to inhibit the growing of *Enterococcus faecalis*, while Silviani and Puspitaningrum (2015) stated that both ethanol and ethyl acetate extracts were able to inhibit enteropathogenic *Escherichia coli* and enterotoxigenic *E. coli*.

Conclusions drawn from this research are: (i) MIC of ethyl acetate extract to *S. aureus* could not be concluded. (ii) MKC of ethyl acetate extract to *S. aureus* was 80%. (iii) The greatest Inhibitory Zone formed was 10.3 mm for 100% concentration. Some suggestion for further research including fractionation test and toxicity tests should be done on active compounds of any extracts.

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# Bioethanol production from rice and corn husks after enzymatic and microbes hydrolysis and yeast fermentation

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**Abstract.** Purwoko Tj, Sari SLA, Mahadjoeno E, Sunarto. 2017. Bioethanol production from rice and corn husks after enzymatic and microbes hydrolysis and yeast fermentation. *Bioteknologi* 14: 19-23. Bioethanol is a renewable resource that can be produced from fermented cellulosic biomass. The use of lignocellulosic materials from agricultural wastes provides a low-cost fermentative substrate. Lignocellulosic ethanol production involves acid or enzymatic hydrolysis. The enzymatic hydrolysis cost higher, however in environmental issue this step is favorable. The purpose of this research was to compare bioethanol production after microbes and cellulosic enzymes hydrolysis following yeast, *Saccharomyces cerevisiae*, fermentation from rice and corn husks. The rice and corn husks (1 kg) were each suspended in water until the volumes reached 5 L. The sample mixtures were treated with 2.5 mg/L cellulases, 5 g/L multienzymes, and 5 mL/L EM4 respectively. The mixtures were stirred for 24 hours at pH 5.7 and 35°C. After hydrolysis, the samples (100 mL) were treated by 1, 2, and 3 % w/v Baker's yeasts. The samples were fermented with incubator shaking for 6 days at 30°C, 90 rpm, and pH 6.5. Sugar concentrations were determined by dinitrosalicylic acid (DNS) colorimetric method. Bioethanol production and specific gravity method were then compared to IAOC Ethanol Table. Sugar concentrations and bioethanol production after multienzymes hydrolysis of rice and corn husks. There were 6.54-6.81 mg/mL and 3.17-3.54 mg/mL, respectively. Sugar concentrations of rice and corn husks after multienzymes hydrolysis treatment was higher than EM4 and cellulases hydrolysis treatments. Therefore, bioethanol productions of rice and corn husks after multienzymes hydrolysis and yeasts after 2 days fermentation were higher than the others.

**Keywords:** Bioethanol, corn husk, multienzymes, rice husk, sugar concentration

## INTRODUCTION

With the energy crisis and increased environmental awareness there has emerged a need for more sources of renewable, the green energy around the world. Bioethanol can be produced through fermentation of sugars. Worldwide interest in the utilization of bioethanol as an energy source has its concern on the efficiency and cost of industrial processes for bioethanol production. Even though the fermentative process of bioethanol production is well known, the production costs are still an important factor for the wide use of bioethanol as a fuel. Therefore, development of fermentation processes, using cheap carbon sources, is important for commercial scale production.

Bioethanol is a renewable resource that can be produced from fermented cellulosic biomass. Bioethanol does not add to a net-CO atmospheric increase. Thus, there is no contribution to global warming. Combustion of ethanol results in relatively low emissions of volatile organic compounds, carbon monoxide and nitrogen oxides (Park et al. 2010), therefore can reduce greenhouse gasses. However, applying bioethanol on gasoline engines involves some modification (Hassan and Kalam 2013). Cost reduction is still essential for large deployment of this new technology. Since the cost of the traditionally used (sugar and starch-containing) raw materials represent a major part of the total production cost (Robelo et al. 2011). Using

fewer valuable materials, like agricultural waste, could reduce the expense significantly.

The use of lignocellulosic materials from agricultural wastes provides a low-cost fermentative substrate. Using agricultural waste as lignocellulosic feedstocks for bioethanol production was greatly promising. One of the advantages of the use of lignocellulosic biomass is not interfering with food production. This implies the production of bioethanol can be done without the need of employing extensions of cultivable land for cropping sugar cane, rice or corn exclusively dedicated to the biofuel production. The development of an innovative waste management approach is focused on using agricultural and industrial waste as cheap substrates. The production of bioethanol by yeast-based sugar fermentation has already been commercially established. Crop wastes are of great interest to reduce the costs of bioethanol production process. Rice and corn are popular crops in Asian countries, including Indonesia. It is essential to hydrolyze lignocellulosic materials before fermentation because yeast cannot use lignocelluloses directly into bioethanol.

Lignocellulosic materials of rice and corn are: rice straw, rice husk, ccorn cobs and corn husk. Cellulose and hemicellulose, which are the principal biodegradable carbohydrate components of the husks, are found together with lignin in an intense cross-linked, rigid lignocellulose complex (Chan 2014). Most reports on lignocellulosic

ethanol production involve acid or enzymatic hydrolysis. Enzymatic hydrolysis is high cost, however this is favorable. Commercial enzymes that apply in poultry can reduce the cost. Moreover, microbes that have cellulolytic activity can also reduce the cost. The objective of this research was to compare bioethanol production after microbes and cellulosic enzymes hydrolysis following yeast *Saccharomyces cerevisiae* fermentation from rice and corn husks.

## MATERIALS AND METHODS

The rice and corn husks were obtained from the farmers at Karanganyar region of Central Java, Indonesia. Baker's yeasts [Fermipan] and microbes [EM4] were obtained from a retail store at Surakarta City (Solo) of Central Java, Indonesia. The cellulases and multienzymes [Naturzime] were obtained from Sukahan, China and Macco Organique Inc., Canada, respectively.

### Sample preparation

The rice and corn husks were sun-drying for 7 days. After drying, the husks were grounded. The ground samples were stored at room temperature ( $\pm 30^\circ\text{C}$ ), and the room humidity was controlled at 50-60% by silica gel. Each of the husk ground samples were treated by hydrolysis and followed by yeasts fermentation.

### Sample hydrolysis

The ground samples (1000 grams) were suspended in water until the volumes reached 5 L. They were then mixed to form a slurry sample. The slurry samples were divided into three samples (1.5 L each). After sterilization by autoclave ( $121^\circ\text{C}$ , 15 min), the slurry samples were treated with 2.5 mg/L cellulases (50 IU), 5g/L multienzymes (50 IU xylanases) and 5 mL/L EM4 respectively. The mixtures were stirred for 24 hours at pH 5.7 and  $35^\circ\text{C}$ . After 24 hours, the mixtures were filtered and stored in a refrigerator at  $4-6^\circ\text{C}$  prior to yeast fermentation.

### Yeast fermentation

The three hydrolysis samples (500 mL each) from hydrolysis were sterilized by autoclaves ( $121^\circ\text{C}$ , 15 min). After cooling, the samples were treated by 1, 2, and 3 % w/v Baker's yeasts. The samples were fermented at incubator shaking [Thermo Scientific] for 6 days at  $30^\circ\text{C}$ , 90 rpm, and pH 6.5.

### Sugar determination

The hydrolysis samples from cellulosic enzymes, Naturzime, and EM4 treatments were determined the sugar concentrations by dinitrosalicylic acid (DNS) colorimetric method. The samples of two, four, and six fermentation days from each hydrolysis treatment previously were also determined the reducing sugar by DNS method.

### Ethanol determination

The fermentation samples at two, four, and six fermentation days each treatment were determined the ethanol by specific gravity methods. The samples (50 mL) were distilled using a rotary evaporator until the distillate samples were reached 10 mL. The distillate samples (10 mL) were measured at  $20^\circ\text{C}$  and converted into bioethanol concentrations by IAOC Ethanol Table. The bioethanol concentrations of fermentation samples were calculated as 20% of bioethanol concentrations of distilled samples.

### Data analysis

Data was analyzed using ANOVA with a 95% confidence level. Reducing sugar from hydrolysis samples were analyzed by 2-way ANOVA with reducing sugar before hydrolysis as covariates. Reducing sugar and bioethanol data from fermentation samples were analyzed by 4-way ANOVA.

## RESULTS AND DISCUSSION

This research used enzymatic hydrolysis of rice and corn husks from microbes (EM4), commercial multienzymes and cellulases. The EM4 is microbe consortium that usually applied on composting or feed fermentation. The microbes in EM4 are *Rhodospseudomonas palustris*, *Lactobacillus casei*, and *Saccharomyces cerevisiae*. *R. palustris* is a photosynthetic gram-negative bacterium and found in wastewaters. *R. palustris* can grow on green-plant derived compounds and can metabolize lignocellulosic and acids, as well as aromatic compounds. *Lactobacillus casei* is found in the human intestine and mouth. The most common application of *L. casei* is industrial, especially for dairy production and probiotics for humans and animals. In the past few years, *L. casei* has azoreductase activity. It means they could degrade azo-bonds completely (Seesuriyachan et al. 2007). *Saccharomyces cerevisiae* is a species of yeast. It has been instrumental to winemaking, baking, and brewing. Yeast cells are found primarily in ripe fruits such as grapes. It is the microbe behind the most common types of fermentation.

Multienzymes are enzymes that are used for increasing food digestion in poultry (Abudabos 2010). Multienzymes consist of galactosidases, galactomannases, xylanases, beta glucanases, amylases, proteases, and phosphatases. Galactosidases, galactomannases, xylanases, beta glucanases and amylases are enzymes that catalyze the polysaccharides hydrolysis of galactosides, galactomannans, xylans, glucans, and starch respectively into simple sugars. Proteases are enzymes that hydrolysis of protein, meanwhile phosphatases remove phosphate groups.

Cellulases are enzymes produced chiefly by fungi, bacteria, and protozoans that catalyze cellulolysis, the decomposition of cellulose and some related polysaccharides. Cellulases break down the cellulose molecule into simple sugars. There are five general types of cellulases based on the type of reaction catalyzed, endo cellulases, exocellulases, cellulbioses, oxidative cellulases and cellulose phosphorylases.

### Sugar concentrations after enzymes and microbes hydrolysis

Sugar concentrations of both rice and corn husk slurries after enzymes and microbes hydrolysis were low (Table 1). Since the substrate complexity, both enzymes and microbes are not easy to degrade both substrates. Both enzymes and microbes could hydrolyze lignocelluloses of rice and corn husks into simple sugars. Therefore, there are increasing sugar concentrations after hydrolysis treatment compared to before hydrolysis treatment. Therefore, sugar concentrations after enzymes and microbes hydrolysis are low, not greater than 7 mg/mL. The responsible microbe in EM4, which hydrolyzed lignocelluloses into oligosaccharides and monosaccharides, was *R. palustris*. However, oligosaccharides are further hydrolyzed into monosaccharides by *L. casei* and *S. cerevisiae*. Lignocelluloses was hydrolyzed into oligosaccharides and monosaccharides by xylanases of the multienzymes. Beta-glucanases and amylases of the multienzymes were hydrolyzed oligosaccharides into monosaccharides.

Table 1 showed that sugar concentrations before hydrolysis treatment were 3.24-4.25 mg/mL. These values were not different significantly ( $p \geq 0.05$ ). Therefore, the statistical analyzed sugar concentrations after hydrolysis treatment did not depend on hydrolysis treatment. Sugar concentrations after multienzymes hydrolysis of rice and corn husks were 6.54-6.81 mg/mL and sugar concentrations after cellulases and EM4 hydrolysis was 5.34-5.65 mg/mL and 5.03-5.45 mg/mL. The sugar concentrations after multienzymes hydrolysis were higher than after cellulases and EM4 hydrolysis ( $p < 0.05$ ). It seemed enzymes of multienzymes were more active to hydrolyzed lignocelluloses in rice and corn husks, than enzymes of EM4 and the cellulases. Sugar concentrations after hydrolysis treatment of rice and corn husks, however, were 5.03-6.81 mg/mL and 5.45-6.54 mg/mL. These values were not different significantly ( $p \geq 0.05$ ). It seemed that lignocelluloses of rice and corn husks had large amounts, therefore the enzyme's activity of multienzymes, EM4 and cellulases on rice and husks were not different.

Sugar concentration of rice husk after multienzymes hydrolysis was 6.81 mg/mL. This value was very low compared to 150 mg/mL and 198 mg/g sugar concentration of rice husk after  $H_2SO_4$  hydrolysis (Novia et al. 2015) and rice straw after hydrochloric acid (HCl) hydrolysis (Hashem et al. 2013) respectively. The lowest sugar concentrations after hydrolysis were due to the small amounts of the multienzymes. However, environment consideration, using enzymes hydrolysis were favorable than acid hydrolysis. This low sugar concentration could be increased if we increased the concentration.

### Sugar concentrations after enzymes and microbes hydrolysis and yeast fermentation

After enzymes and microbes hydrolysis, all samples were fermented using yeast *S. cerevisiae* for six days. Sugars are consumed by yeasts for growth and energy generation. Sterilization stopped the enzymatic and microbes activities. Therefore, there is no hydrolysis of lignocelluloses to sugar. The yeast *S. cerevisiae* lacked

lignocelluloses hydrolytic enzymes. However, the yeast had disaccharides hydrolytic enzymes, galactosidases or maltases. Therefore, there is no additional sugar concentration during fermentation, except hydrolysis of disaccharides into monosaccharides. This condition was directed to the decreasing sugar concentrations following increasing fermentation days (Table 2). Sugar concentrations of two fermentation days were 2.42-7.28 mg/mL and higher than sugar concentrations of four (1.88-6.66 mg/mL) and six (1.42-5.71 mg/mL) fermentation days ( $p < 0.05$ ). Decreasing sugar concentrations following increasing fermentation days were due to the metabolism of the yeasts. The yeasts used sugars for growth and generating energy.

Sugar concentrations of the rice and corn husks after multienzymes hydrolysis and yeast fermentation were 1.42-4.63 mg/mL. There were lower than sugar concentrations of the rice and corn husks after EM4 (3.82-7.28 mg/mL) and cellulases (4.49-6.66 mg/mL) hydrolysis and yeast fermentation ( $p < 0.05$ ). The low sugar concentrations of the rice and corn husks after multienzymes hydrolysis and fermentation due to the higher sugar consumption by yeast *S. cerevisiae*. Higher sugar concentration at the beginning of fermentation would stimulate yeast *S. cerevisiae* to uptake sugars into the yeast cells. Sugar concentrations after hydrolysis and fermentation of rice and corn husks were 1.71-7.28 mg/mL and 1.42-6.95 mg/mL. These values were not different significantly ( $p \geq 0.05$ ). This showed that sugar concentrations at beginning fermentation were similar, therefore yeast *S. cerevisiae* activities on sugar of rice and corn husks were similar too.

### Bioethanol productions after hydrolysis variation and fermentation

According to Lin et al. (2012) in aerobic condition, yeast cells were initially grown in fermentative metabolism to produce ethanol, and then yeast cells were consumed ethanol in respiratory metabolism. The anaerobic condition was applied to our research, therefore there is no respiratory metabolism, instead of fermentative metabolism. Ethanol was the 'off product' when yeast consumed sugar for generating energy in fermentative metabolism. Ethanol was expelled from the yeast cell after produce; therefore, we could ease to isolate it. However, yeast *S. cerevisiae* could consume ethanol when glucose concentrations were very low (Raamsdonk et al. 2001).

**Table 1** Sugar concentration (mg/mL) of rice and corn husks after enzymes and microbes hydrolysis.

Hydrolysis treatment		Rice husk	Corn husk
Before hydrolysis	EM4	3.36	3.24
	Multienzymes	4.13	3.65
	Cellulases	4.25	3.40
After hydrolysis	EM4	5.03a	5.45a
	Multienzymes	6.81b	6.54b
	Cellulases	5.34a	5.65a

Note: a,b: the numbers following different notations are different significantly at same columns ( $p < 0.05$ ).

**Table 2.** Sugar concentrations (mg/mL) of rice and corn husks after yeasts fermentation.

Agricultural waste types	Hydrolysis treatments	Yeast conc.	Fermentation days		
			2-days	4-days	6-days
Rice husk	EM4	1%	7.28a,x	4.23a,y	4.07a,y
		2%	6.83a,x	5.14a,y	3.87a,y
		3%	7.25a,x	4.80a,y	4.12a,y
	Multienzymes	1%	3.76b,x	1.88b,y	1.42b,y
		2%	2.42b,x	2.31b,y	1.71b,y
		3%	2.70b,x	2.29b,y	1.76b,y
	Cellulases	1%	6.64a,x	6.66a,y	5.45a,y
		2%	6.01a,x	5.45a,y	5.10a,y
		3%	6.19a,x	5.34a,y	5.71a,y
Corn husk	EM4	1%	6.87a,x	4.23a,y	3.82a,y
		2%	6.95a,x	4.83a,y	3.87a,y
		3%	6.90a,x	4.63a,y	4.12a,y
	Multienzymes	1%	4.63b,x	2.00b,y	1.42b,y
		2%	3.62b,x	2.31b,y	1.71b,y
		3%	3.33b,x	2.29b,y	1.76b,y
	Cellulases	1%	6.16a,x	5.71a,y	5.16a,y
		2%	5.64a,x	5.45a,y	4.49a,y
		3%	5.79a,x	5.26a,y	4.69a,y

Note: a,b: the numbers following different notations were different significantly at same columns ( $p < 0.05$ ). x,y: the numbers following different notations were different significantly at same rows ( $p < 0.05$ ).

**Table 3** Bioethanol concentration (mg/mL) of rice and corn husks after hydrolysis and yeast fermentation.

Agricultural waste types	Hydrolysis treatments	Yeast conc.	Fermentation days		
			2-days	4-days	6-days
Rice husk	EM4	1%	3.35a	3.17a	4.22a
		2%	3.49a	3.19a	3.08a
		3%	3.54a	3.28a	3.14a
	Multienzymes	1%	3.17b	3.33b	3.45b
		2%	3.31b	3.54b	3.17b
		3%	3.31b	3.71b	3.24b
	Cellulases	1%	3.43a	3.24a	3.38a
		2%	3.14a	3.10a	3.19a
		3%	3.21a	3.26a	3.52a
Corn husk	EM4	1%	3.28a	3.42a	3.21a
		2%	3.40a	3.49a	3.28a
		3%	3.39a	3.40a	3.24a
	Multienzymes	1%	3.14b	3.28b	3.26b
		2%	3.47b	3.59b	3.40b
		3%	3.28b	3.54b	3.42b
	Cellulases	1%	3.24a	3.24a	3.28a
		2%	3.33a	3.24a	3.24a
		3%	3.28a	3.26a	3.19a

Note: a,b: the numbers following different notations are different significantly at same columns ( $p < 0.05$ ).

Bioethanol productions after hydrolysis and fermentation of two (3.14-3.54 mg/mL), four (3.10-3.71 mg/mL) and six (3.08-4.22 mg/mL) days were similar and not different significantly ( $p \geq 0.05$ ). It seems that yeast *S. cerevisiae* fermented sugar into ethanol quickly and gave

maximum bioethanol productions. Then yeasts slow their fermentation and consumed ethanol and convert into esters (Peddie 1990), therefore decreasing but not significantly bioethanol production. These esters were responsible for wine flavor.

Bioethanol production after hydrolysis and fermentation of rice husks were 3.08-4.22 mg/mL and did not differ significantly with after hydrolysis and fermentation of corn husks (3.14-3.59 mg/mL;  $p \geq 0.05$ ). This showed bioethanol production was not different at rice and corn husks. These showed that similar sugar concentrations at beginning fermentation (see Table 2) would lead similar bioethanol productions of rice and corn husks.

Bioethanol production after multienzymes hydrolysis of rice husk was 3.17-3.54 mg/mL. However, the highest bioethanol production of rice husk was 4.22 mg/L and obtained after EM4 hydrolysis. These bioethanol productions were lower than bioethanol obtained by Hashem et al. (2013) and Rabah et al. (2014). They were obtained 6.31 mg/L and 5.8 mg/L bioethanol from rice straw and rice husk respectively. This showed bioethanol productions mainly depend on the sugar concentrations. Sugar concentration in our research was lower than Hashem et al. and Rabah et al., therefore bioethanol production was lower too.

Bioethanol production after multienzymes hydrolysis of corn husk was 3.59 mg/mL and lower than 10.08 mg/mL that obtained by Itelima et al. (2013) who used co-fermentation *Aspergillus niger* and *S. cerevisiae* on corn cobs. However, when corn cobs were hydrolyzed by *Aspergillus niger* and then fermented by *Saccharomyces cerevisiae*, the ethanol production was only 0.64 mg/mL (Zakpa et al. 2009). This showed hydrolysis by *A. niger* at the same time with and fermentation by yeast *S. cerevisiae* would give continuous sugar to yeast and then yeast ferment sugar to bioethanol. However, this was not evident when hydrolysis by *A. niger* was at a different time with and fermentation by yeast *S. cerevisiae*.

We could also compare sugar consumption and bioethanol production. We did assume that when sugars consumptions were high, then bioethanol productions were high too. This assumption was true when we compared Table 2 and Table 3. In Table 2, yeast at multienzymes hydrolysis treatment consumed sugars higher than at EM4 and cellulases hydrolysis treatments. In Table 3, yeast at multienzymes hydrolysis treatment produced bioethanol higher than at EM4 and cellulases hydrolysis treatments. If we compared sugar consumption and bioethanol production, we then saw that sugar consumption was two times higher than bioethanol production. This comparison corresponded with the theoretical calculating of producing bioethanol from sugar.

In conclusion, sugar concentrations of rice and corn husks after multienzymes hydrolysis treatment was higher than EM4 and cellulases hydrolysis treatments were 6.54-6.81 mg/mL. Therefore, bioethanol productions of rice and corn husks after multienzymes hydrolysis and yeasts after 2 days fermentation were 3.17-3.54 mg/mL.

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