Potential Test of Endophytic Yeast From Sweet Oranges (*Citrus sinensis* L.) as Leavening Agent For Bread

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Abstract. Yeast is one of the most important ingredients in bread making and, it may be isolated from local fruits such as sweet oranges as a commercial yeast substitute. This research aimed to isolate yeast from sweet orange (Citrus sinensis L) that may be used as a bread improver. The method used was the endophytic yeast isolation on the rind and flesh of sweet citrus fruits. It is identified macroscopically, microscopically and analyzed for carbohydrate fermentation, growth in 50% glucose medium, hydrogen sulfide (H_2S), flocculation, temperature tolerance, and ethanol, as well as the use of yeast isolates as bread dough improver. Five yeast isolates were obtained based on the results: RYB1, RYB2, RYK1, RYK2, and RSK1. All isolates had similarities with the Ascomycetes class, specifically the Hemiascomycetes subclass, as indicated by the overall asexual reproduction of yeast cells by budding. RYK2 isolate was the best bread improver, with the same dough volume as the positive control (Fermipan®) and a better aroma than the positive control. Subsequently, Isolates RYB1, RYB2, and RSK1 had a satisfactory ability to develop bread dough, unlike RYK1. Furthermore, the RYK2 isolate has the highest potential for bread development and can be a commercial yeast candidate.

INTRODUCTION

The existence of the world's bread industry, including in Indonesia, from time to time is always developing. In Indonesia, commercial baker's yeast is still imported from France (Saf-instant®) and Canada (Fermipan®). In recent years the idea of natural yeast agents has increased as a form of innovation in replacing commercial baker's yeast [1]. Yeast has an important role in increasing flour dough through the carbohydrate fermentation process [2]. Yeasts used in the fermentation process can be isolated naturally from natural materials, such as water, soil, fruits and flowers or in other words are usually referred to as endophytic yeasts [1].

Sweet orange (Citrus sinensis L.) as a local fruit native to Indonesia and recorded as having the highest position in the agro-industry sector, both used for consumption of fresh fruit and in processed products [3]. Some yeasts that have potential as bread developers are *Candida parapsilosis, Candida stellata, Saccharomyces serevisiae, Torulaspora delbrueckii* and *Zygosacchomyces rouxii* [2]. In addition, typical species that can be found in sweet citrus fruits are *Saccharomyces exugus* and *Saccharomyces ludwigii*, *Torulaspora delbrukii*, *Geotrichum capitatum, Kodamaea ohmeri*, several species from *Rhodotorula, Pichia, Hansenispora* and *Metschickowia* [4]. *Saccharomyces cerevisiae* is one of the yeast microorganisms which, according to research, is found in orange peels. High glucose and pectin levels are what support yeast growth and make it easier for yeast to associate with orange peel [5].

Yeast has four roles in the bread-making process, namely to increase the volume of the dough due to the formation of carbon dioxide during the fermentation process, to develop the structure and texture of the dough due to stretching by the expansion of CO2 gas bubbles, to improve the taste and to add some nutritional value to the bread [4]. Yeast for bread developers must have basic requirements, namely being able to survive in conditions that have high ethanol levels between 10%-15%, and tolerance to various temperature conditions of 25 ^oC, 30 ^oC, 37 ^oC dan 45 ^oC [6]. Good

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yeast as a developer Bread must be able to form flocculants or floc deposits in the flocculation process and not produce hydrogen sulfide (H_2S) gas which is a compound that forms a foul smell and bad taste in bread processing [2].

Analysis of yeasts from sweet oranges is limited to isolation and identification of species, especially in Indonesia, research on the isolation and application of yeast on fruit, especially sweet oranges, has almost never been carried out in Indonesia. This study aims to isolate and analyze the potential of sweet orange endophytic yeast as a bread dough developer. The hope of this research is to be able to obtain competent isolates that can be developed into commercial yeast.

METHOD

The materials used in this study were sweet orange (*Citrus sinensis* L.) from Banaran, Bumiaji District, Batu City, media *Yeast Medium* (YM), *Glucose Yeast Peptone* (GYP), *Yeast Malt Extract Agar* (YMA), *Sodium DL-Lactate*, *Yeast Malt Extract Agar Broth* (YMB)**,** bread dough development media (flour, sugar, salt, margarine and water) and Fermipan® (Commercial yeast).

Yeast Malt Extract Agar/ Broth (YMA/B) Media

YMB media was made from 5 grams of peptone, 10 grams of glucose, 3 grams of yeast extract, 3 grams of malt extract dissolved in 1000 ml of distilled water, while to YMA media was added 20 *microbial agar*. Then the media was sterilized in an autoclave at 121 ºC. If the media temperature is approximately 50 ºC, then add *Sodium DL-Lactate* which is useful as an antibacterial as much as 120 µl [7].

Isolation and Purification Yeast

The orange peel was cleaned using 70% alcohol and then dried. Sweet oranges are washed, cut into 3x3 cm using a sterile knife and separated from the skin. Yeasts associated with citrus fruits were isolated by soaking in YMB media with added *Sodium DL-Lactate* as an antibacterial. Immersion was carried out in a 50 ml Ependorf tube. Incubation was carried out for 3 days at room temperature or until air-filled bubbles were formed. The presence of bubbles indicates that fermentation has occurred in the media due to yeast growth and development [8].

The yeast that had grown on YMEB media was then diluted. Inoculation was carried out on the results of the 10^{-3} dilution by *spread plate* on YMEA media. Next, incubation was carried out at room temperature (27 ºC) for 48 hours [9]. Colonies that looked different from other colonies from morphological characters and media, were inoculated on YMEB media as much as 3 ml in a test tube and incubated for 7 x 24 hours in a shaker incubator until looks cloudy. Growth results in YMEB media spread plate on YMEA media. The second and third subcultures of yeast were carried out by streak plate on YMEA media until pure isolates were obtained. When pure colonies were obtained, streak plates were performed on an YMEA slant.

Identification Macroscopic and Microscopic

The purified yeast was observed for macroscopic characters on YMEA media in petri dishes. The macroscopic characters observed included the surface, edges or margins, texture, color and elevation of the colony. While microscopic observations were in the form of size, shape and means of vegetative reproduction such as the presence or absence of budding formation in yeast cells [10].

Bread Developer Potential Analysis

Carbohydrate fermentation test

The carbohydrate fermentation test was carried out by adding 100 µl of yeast with 48 hours of yeast age to 9 ml of the fermentation test medium (glucose, lactose, sucrose and fructose). Then it was incubated at 27 ºC for 7 days. The fermentation medium was put in a test tube equipped with a Durham tube [6]. A positive reaction for fermentation was indicated by a change in the media from red to pink followed by the formation of bubbles in the Durham tube.

While the negative reaction was indicated by the absence of bubbles in the Durham tube and no change in the color of media [9].

Growth test on 50% glucose medium

Growth test on glucose levels was carried out with reference [11]. Yeast isolate was inoculated in 5 ml of YPG medium in a test tube with a glucose concentration of 50% (m/v) and incubated for 48 hours at 25 oC. Yeast cell density was analyzed using Spektrofotometer *UV-vis*. A positive reaction was indicated by a high number of yeast cell density.

Hydrogen sulfide (H_2S) and flocculation test

Yeast isolates were grown on *lead acetate* media (40 g/L glucose, 5g/L *yeast extract*, 3 g/L peptone, 0.2 g/L ammonium sulphate, 1 g/L *lead acetate* and 20 g/L agar) and incubated. at a temperature of 30 oC for 7 days [6]. The color change of the bottom of the media to black indicates the formation of hydrogen sulfide [12]. The flocculation test was carried out by means of yeast isolates inoculated on 10 ml YPG media and incubated in a shaker incubator for 3 days at 30ºC Yeast isolates that have gone through the incubation process are centrifuged at a speed of 5000 rpm for 10 minutes [2]. This test is carried out to observe the formed flocculation, the ability of yeast in this flocculation test is indicated by the formation of flocculants or the presence of a layer in the tube.

Temperature and ethanol tolerance test

Temperature tolerance test was carried out by means of yeast isolates grown on YPG agar media and incubated at 25 ºC, 30 ºC, 37 ºC dan 45 ºC for 72 hours. While the ethanol tolerance test was carried out with yeast isolates in YPG broth media containing 3 different concentrations of ethanol, namely 10%, 13% and 15%. It was then incubated at 30 ºC for 72 hours. The density of yeast cells was calculated after and before the incubation process took place using a Spektrofotometer *UV-vis* at a wavelength of 660 nm [6].

Application of isolate as a bread dough developer

The application of the isolate as a bread dough developer was carried out by growing yeast strains in 15 ml Ependorf tubes in YPG media and then put in an *incubator shaker* for 72 hours at 30 ºC. To obtain yeast pellets which were then used in the development of bread dough, centrifugation was carried out at 10,000 rpm for approximately 5 minutes. Pellets or flocs formed at the base of the media are then used as dough developers [6].

The potency test for each yeast strain, prepared 50 grams of flour, 1.5 grams of salt mixed with flour, 3 grams of sugar dissolved in 50 ml of warm water and 0.6 grams of yeast pellets obtained from the results of centrifugation, then all the ingredients were mixed. and stirred evenly. This test is equipped with a positive control test (Fermipan®) and a negative control in the form of bread dough without added yeast. The dough is left at 37 ºC with an interval of 1-12 hours. The development of the dough was observed during incubation, the volume after baking was measured and organoleptic test was carried out [2].

RESULT AND DISCUSSION

Isolation and Identification Yeast of Sweet Citrus

Isolation of yeast on sweet orange (*Citrus sinensis* L.) obtained five isolates. Three isolates from peel with isolate code RYK1, RYK2 and RSK1 and two isolates from pulp fruit with isolate code RYB1 and RYB2. The results of observations of macroscopic and microscopic characters can be seen in **Table I** and **Figure 1**.

TABLE I. Macroscopic and microscopic characters of yeast isolates

Isolate	Edge	Elevation	Colony shape	Cell shape	Reproduction	Size ℓ x p (µm)
RYB1	Uneven	Average	Round	Oval	Monopolar	4,08 x 7,76
RYB2	Average	Arise	Round	Oval	Monopolar	4,31 x 6,41
RYK1	Uneven	Arise	Irregular	Oval	Monopolar	6,94 x 7,10
RYK2	Average	Average	Round	Oval	Multilateral	5,52 x 7,36
RSK1	Average	Arise	Round	Oval	Monopolar	4,42 x 4,70

All yeast isolates performed asexual reproduction by *budding* (**Table I**). Budding can be monopolar (1 pole), bipolar (2 pole) and multilateral (many poles). The poles in question are daughter cells that are formed following the body of the parent cell. Yeast cell sizes range from wide 4-6 µm and long 6-8 µm [13]. Based on macroscopic and microscopic identification, the two groups of isolates had similarities with the *Ascomycetes* class of yeasts, *Hemiascomycetes* subclasses. The subclass *Hemiascomycetes* has a cell wall that consists of two layers and performs asexual reproduction by *budding*. Yeasts in the *Hemiascomycetes* subclass are yeasts that are often associated with the food processing industry [14].

Carbohydrate Fermentation Test

Carbohydrate fermentation test showed that all isolates were able to ferment carbohydrates well in glucose and fructose types, especially RYK2 isolates (**Table II**). All yeast isolates were unable to ferment the type of sugar lactose, which was indicated by the absence of bubbles. *Saccharomyces cerevisiae* is one of the yeast breading agents that cannot ferment lactose because of the lack of enzymes such as lactase and β-galactosidase [15].

TABLE II. The ability of yeast isolates to ferment carbohydrates on the 7 days and changes in pH

Isolate	Sugar fermentation test											
	Glucosa			Sucrosa			Fructosa			Lactosa		
	colour	gas	pH	colour	gas	pH	colour	gas	pH	colour	gas	pH
RYB 1	Pink	+	6	Red	-	7	Pink	+	8	Red	-	7
RYB 2	Pink	+	7	Red	-	7	Pink	+	7	Red	-	7
RYK 1	Pink	+	5	Red	-	7	Pink	+	5	Pink	-	5
RYK 2	Pink	+	7	Pink	+	5	Pink	+	6	Red	-	7
RSK 1	Pink	+	6	Pink	-	7	Pink	++	6	Red	-	7
Control	Pink	-	7	Red	-	7	Pink	-	7	Red	-	7

Notes: (-) no bubbles are formed; (+) slight bubbles; (++) lots of bubbles

Tests of carbohydrate fermentation on the type of fructose sugar showed that all yeast isolates were able to ferment well, marked by the formation of bubbles with high intensity in RSK1 isolates. The formation of CO_2 gas in the Durham tube is due to the activity of enzymes that play a role in alcoholic fermentation. Enzymes in the form of invertase, zimase, carboxylase, hexokinase and dehydrogenase are enzymes produced by yeast to help the fermentation process. One of the enzymes that acts as a biocatalyst is the zimase enzyme, this enzyme works by converting glucose and fructose into alcohol and CO_2 [16].

Some isolates on certain types of sugar experienced changes in pH from 7 to 5-8. pH or degree of acidity is one of the important factors affecting the growth of microorganisms and the formation of products in the fermentation process. On the 5th day there was an increase in pH due to yeast experiencing a growth phase, so that the conversion of sugar into ethanol occurred quickly. The impact that occurs from the growth phase is that it can increase the OH-group so that the pH increases to alkaline (C_2H_5OH). While the pH of fermentation on days 6 and 7 decreased, because during the fermentation process it produces dissolved CO_2 gas which is acidic (H_2CO_3). As a result of organic acids formed from fermentation by-products will result in a decrease in pH. In general, yeast can grow optimally at a pH of 4-6.8 [17].

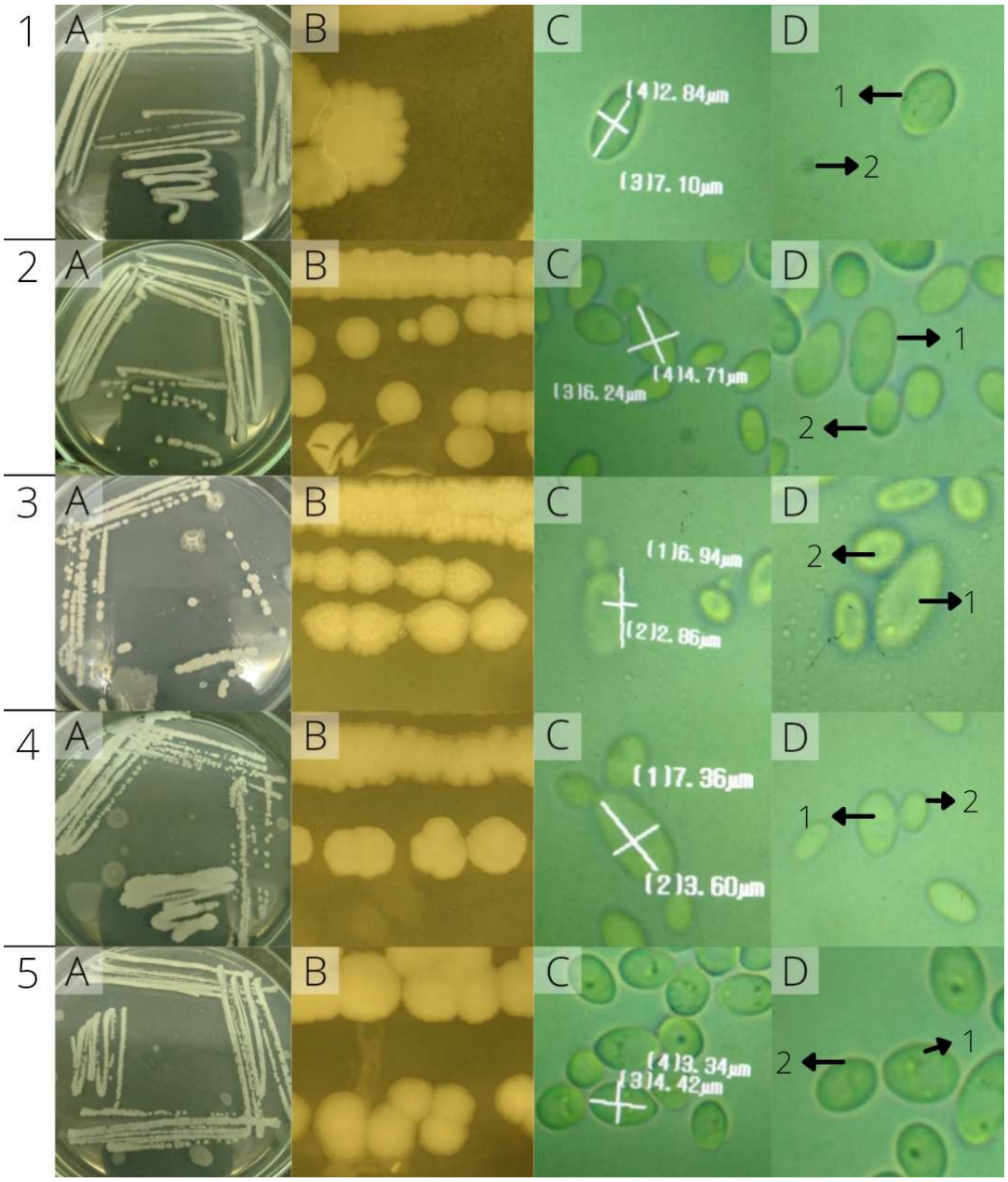

FIGURE 1. Isolate 1) RYB1; 2) RYB2; 3) RYK1; 4) RYK2; 5) RSK1, a) Colony in YMEA media b) Colony 10x magnification, c) Cell shape 1000x magnification, d) 1. Stem cells 2. Stem cells, monolateral budding type.

Yeast ability test on 50% glucose

All yeast isolates found to grow well in high sugar conditions based on the calculation of the optical density (OD) value were indicated by the increase in the number of yeast cell density after 48 hours of incubation (**Table III**). Yeast isolate which was found to have potential as a yeast dough developer, Yeast isolate used as a bread dough developer was able to grow well at a glucose concentration of 20% (m/v) in a qualitative test [15]. At the beginning of the fermentation process with appropriate levels, yeast is able to tolerate osmotic pressure by utilizing existing sugars and synthesizing glycerol and producing low levels of acid, but the presence of high sugar content in the yeast growth medium produces high osmotic pressure so that it will inhibit the growth process yeast [18]

TABLE III. Growth of yeast cell isolates on 50% (m/v) glucose medium

Isolate	Glucosa 50% (m/v)	
	24 h	48 h
RYB1	0.113	0.385
RYB2	0.276	0.342
RYK1	0.125	0.281
RYK2	0.124	0.284
RSK1	0.147	0.326
Control	0.000	0.000

H_2S Formation and Flocculation Test

Based on the results of the study, out of five yeast isolates there were two isolates that did not respond to the H_2S test, namely, RYB2 and RYK2 isolates and controls (**Table IV**). The formation of H_2S is indicated by the presence of a black precipitate on the surface of the medium. Black precipitate as indicator of hydrogen sulfide in *Lead Acetate Agar* media occurs due to reduction of inorganic sulfur thiosulfate [12]. Hydrogen sulfide (H_2S) is a compound associated with an unpleasant aroma and taste in food processing [2]. Yeast isolates showing high H_2S production are undesirable for bread making because they give bad taste and interfere with bread quality. However, in some studies it is stated that the presence of H_2S can be tolerated to a certain level. On research Asyikeen *et al*., (2013) [15] showed that all yeast isolates produced H_2S including isolates that were considered superior. In addition, in his research, commercial yeast used as a positive control also showed high H_2S production so that the yeast isolates found were acceptable as bread dough expanders. This indicates that H_2S levels are acceptable in food products, in accordance with the circumstances and conditions that occur.

TABLE IV. H_2S formation test and flocculation formation

Isolate	H_2S	Floculasi
RYB1	++	+
RYB 2	-	++
RYK 1	+	+
RYK 2	-	+
RSK 1	+	+
Control	-	-

Notes : (++) intensive response, (+) normal response, (-) no response

All isolates were able to form flocculants on the bottom of the media (**Table IV**). The ability of yeast isolates to form flocculants in the flocculation process is also an important factor to determine as a candidate for baker's yeast. Yeast isolates which have the ability to form flocculants in the flocculation process are due to cell adhesion forces and are an important requirement for yeast in the bread making industry. The principle of the flocculation process occurs due to the separation of yeast cells and the medium in which they grow, so that flocculants or pure yeast cells are formed which can then be produced for commercial yeast in the industrial sector. From the flocculants formed, it shows that yeast isolates from the skin and flesh of sweet oranges have potential as bread dough developers [15].

Yeast Tolerance Test for Temperature and Ethanol

Based on the optical density, all yeast isolates were able to grow optimally at 30 ^oC (**Table V**). The value of cell density increased after 72 hours of incubation. Yeast isolates that were able to grow at higher temperatures were good isolate candidates as bread dough developers. In addition, isolates that are tolerant of high-temperature environmental conditions have better potential in increasing the production of carbon dioxide gas in the fermentation process [6]. The importance of the ability of yeast to grow in conditions of uncertain temperature will be related to the fermentation and incubation processes. In general, yeasts are able to grow at room temperature 26-37 ^oC [2]. Different things were said by [11], optimal conditions for yeast growth are 25-30 ^oC, humidity and temperatures that are too hot or too cold can slow down yeast growth and will be related to yeast quality.

All yeast isolates obtained showed good growth with alcohol content of 10, 13 and 15% (**Table VI**). The growth of yeast isolates was indicated by the increase in the number of yeast cell density after incubation for 72 hours. Not

many yeasts have the ability to tolerate excess alcohol. In general, yeast can grow optimally in an alcohol content of 10%. Excess alcohol content generally inhibits the fermentation process, because high alcohol conditions can affect the osmotic pressure/fluidity of the membrane so that it interferes with yeast growth [18]. Alcohol with high enough levels can damage mitochondrial DNA and cause enzyme inactivation and damage cell membranes, resulting in yeast death [6]. The ability of yeast that can grow at high alcohol concentrations can increase the aroma of bread dough [15].

TABLE V. The ability of yeast isolates in temperature tolerance

Isolate	30 ºC		37 ºC		45 ºC	
	24 h	72 h	24 h	72 h	24 h	72 h
RYB 1	1.615	3.236	0.732	1.824	0.087	0.613
RYB 2	1.889	2.556	1.323	2.360	0.104	0.604
RYK 1	1.873	3.170	0.452	2.289	0.108	0.902
RYK 2	0.884	2.930	0.423	1.870	0.458	0.995
RSK 1	0.789	2.786	0.253	1.146	0.067	1.195
Control	0.000	0.000	0.000	0.000	0.000	0.000

TABLE VI. The ability of yeast isolates to tolerate alcohol

Isolate	10 %		13 %		15 %	
	24 h	72 h	24 h	72 h	24 h	72 h
RYB1	0.260	0.920	0.344	0.401	0.018	0.355
RYB2	0.234	0.652	0.137	0.170	0.004	0.181
RYK1	0.268	0.311	0.072	0.554	0.158	0.771
RYK2	0.076	0.148	0.304	0.500	0.153	0.605
RSK1	0.043	0.217	0.121	0.157	0.134	0.450
Control	0.000	0.000	0.000	0.000	0.000	0.000

Application of isolate as a bread dough developer

The expansion of the dough from RYB2 and RYK2 isolates began to expand to fill the bottom of the incubation glass, followed by RYB1 isolates at 6 hours. However, when compared to the positive control the development of isolates was found to be still far behind. This was because the positive control yeast (Fermipan®) contained the emulsifier sorbitan monostearate E491. According to [19], Sorbitan monostearate is a food additive used in the manufacture of food products and is a non-ionic surfactant with emulsifying, dispersing and wetting properties. In the manufacture of bread, sorbitan monostearate emulsifier functions to improve and increase the volume of the bread so that it expands and maintains the stability of the texture of the bread when it is in cold temperature conditions. In addition, the addition of lactic acid bacteria in the form of *Lactobacillus bulgaricus* can extend the shelf life of bread, increase the volume and aroma of bread [20].

Incubation of bread dough for 12 hours showed that the entire dough experienced expansion when compared to positive and negative controls (**Figure 2**). RYK2 isolate had the highest development rate compared to other isolates. The development that occurs can be seen from the increase in the volume of the dough as a result of the ongoing fermentation process. The fermentation process that takes place produces CO_2 gas so that air cavities are formed and development occurs in the dough. Yeast works by utilizing sugar to produce ethyl alcohol and carbon dioxide (CO_2) to develop bread dough. The CO_2 gas that is formed will be retained by gluten from the flour so that it will form a dough that expands with air cavities [21].

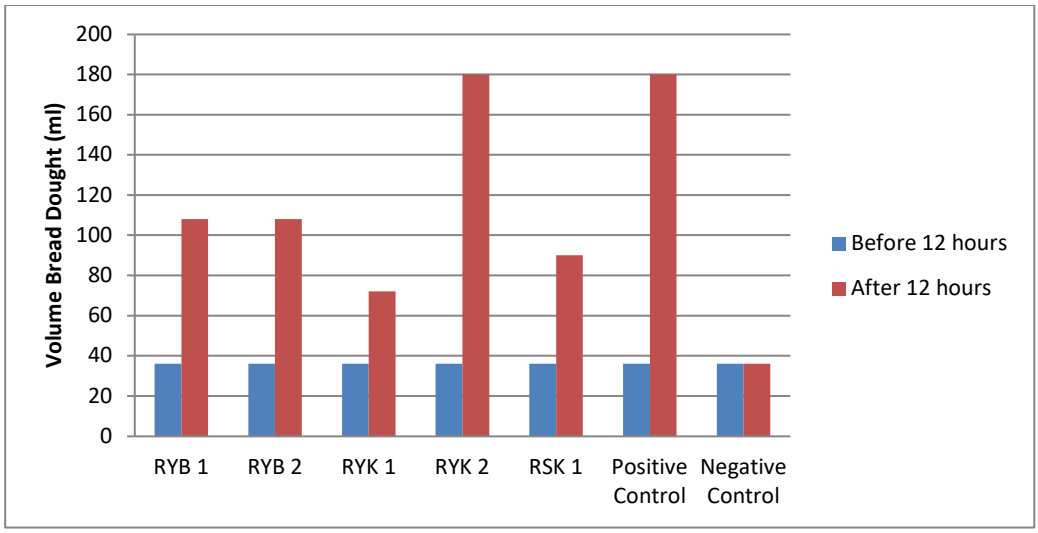

FIGURE 2. Volume bread dought after 12-hour incubation

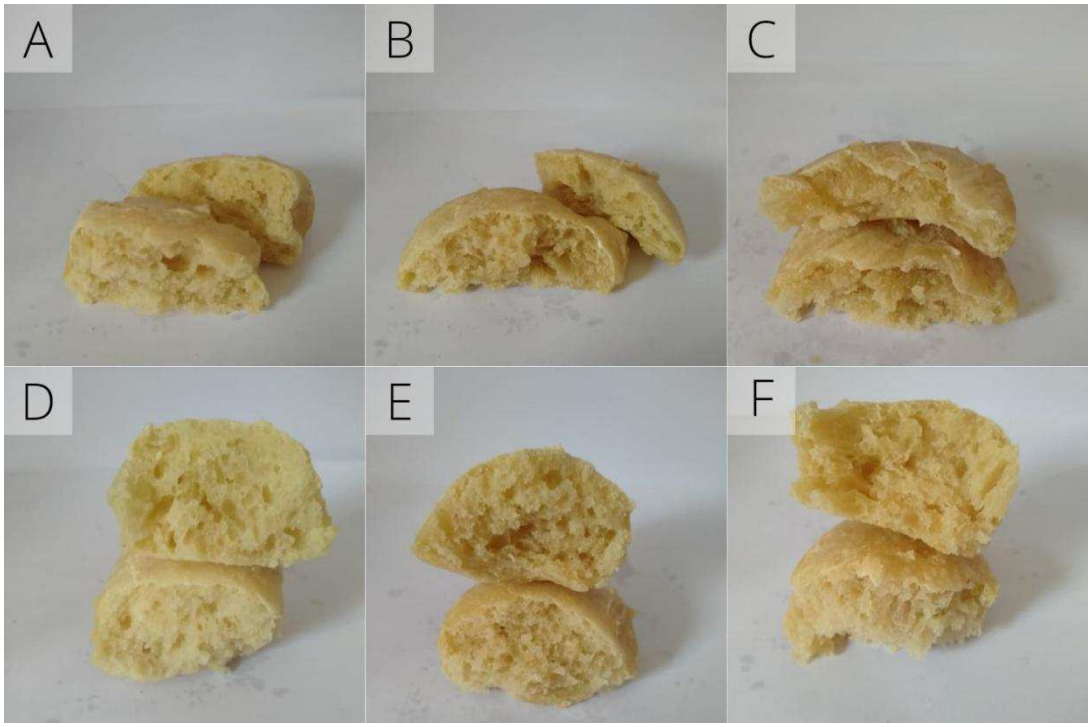

FIGURE 3. Toast cavity, (a) RYB1 isolate; (b) RYB2 isolates; (c) RYK1 isolates; (d)RYK2 isolates; (e) RSK1 isolate; (f) positive control (Fermipan®)

All isolates had the ability to increase the dough volume. During the fermentation process, yeast cells will divide and utilize gluten protein from flour to then produce cavities due to the presence of carbon dioxide formed from the breakdown of carbohydrates from the substrate where yeast lives. From the air cavity that is formed, the bread dough experiences an increase in the volume of air so that the dough shows an expansion [22].

The baking process of bread dough showed that RYK2 isolate (**Figure 3**) had a superior size of expansion compared to other isolates. During the baking process there will be an increase in the volume of dough due to high

heat pressure. Characteristics of bread after baking are presented in **Table VII**. Bread from RYK2 isolate had a more fragrant aroma than bread with positive control ((Fermipan®). RYK2 isolate had a creamy color with lots of cavities on the inside of the bread resembling positive control and hard texture. on the outside and soft on the inside. Bread from isolates RYB1 and RYB2 has almost the same characteristics, namely it has a cream color with a distinctive fermented aroma and a cavity is formed on the inside of the bread and the outer texture of the bread is hard and soft on the inside.

TABLE VII. Characteristics of bread after baking from yeast isolate

Isolate	Colour	Aroma	Textur	
			out	inside
RYB 1	Cream	++	Hard	Soft hollow
RYB 2	Cream	++	Hard	Soft hollow
RYK 1	Brown	+	Hard	Not hollow
RYK 2	Cream	+++	Hard	Soft hollow
RSK 1	Cream	++	Hard	Soft hollow

Notes: (+) Not Good; (+) Good; (++) Very Good

The color change in the dough after the oven process that occurs is related to the ingredients used such as the use of sugar. The presence of sugar in the manufacture of bread serves as an energy source for yeast. The sugar residue that is not used up in the fermentation process will give a sweet taste and a creamy or *golden brown* color to the bread. In addition, sugar also plays a role in the process of coloring the outer skin of the bread in the oven baking [23]. In addition, aroma is the second important factor after color because in general the consideration in the acceptance of a food ingredient is based on the assessment of aroma. Texture is no less important in making bread. In addition to the presence of yeasts that ferment to produce CO_2 and alcohol, the use of flour plays an important role in the resulting texture [24].

CONCLUSION

On the skin and flesh of the sweet orange fruit, five yeast isolates were found which were coded RYB1, RYB2, RYK1, RYK2, and RSK1. All isolates had similarities with the Ascomycetes class, Hemiascomycetes subclass, which was characterized by the overall asexual reproduction of yeast cells using *budding*. RYK2 isolate had the best ability as a bread developer based on biochemical tests. In addition, the dough volume was the same as the positive control (Fermipan®), and had a better aroma than the positive control. Isolates RYB1, RYB2, and RSK1 had good ability, while RYK1 did not have good ability to develop bread dough.

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