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A new source of *Saccharomyces cerevisiae* as a leavening agent in bread making

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Abstract

Megabiodiversity of Malaysian's flora and fauna which include microorganism could be conserved and served as alternative source indigenous yeast, the leavening agent of commercial bread making. This study was conducted in attempt to exploit the potential of *Saccharomyces cerevisiae* strains isolated from 30 different local fruits and plant parts as a leavening agent in bread making. The enrichment was carried out by fermenting the plant samples in medium containing Grape Must at 25°C for 10 days following by isolation of tentative yeasts at 30°C for 3 to 5 days. 20 out of 30 samples tested showed the presence of yeasts was then selected for identification of *S. cerevisiae* strains through biochemical and physiological tests. Of the 20 yeast strains examined, 13 strains were identified as *S. cerevisiae* and potentially used as leavening agent in bread making where 5 strains namely SN3, SMK9, SDB10, SRB11 and SS12 showed better fermentative performance compared to commercial strains. Thus, indicated that the local fruits and plant parts could be the potential source of indigenous *S. cerevisiae* strains for leavening agent in bread making.

Introduction

As a predominant species it is well known that *S. cerevisiae* plays an important role as a leavening agent in bread making. The leavening step is essential in the fermentation of dough. It does not only induce and increase the volume of dough through gas incorporation but helps creating the desired flavor and texture (Fleury *et al*., 2002).

Previous researcher have argued that *S. cerevisiae* does not live in other environment except nature, but can be found only in vinery environment as a wild or domesticated species (Martini *et al*., 1993). In many instances, the strains are clearly specialized for use in the laboratory (Mortimer and Johnston, 1986) and in the production of wine (Kunkee and Bisson, 1993), beer (Hammond, 1993) and bread (Rose and Vijayalakshmi, 1993). This has lead the common view that *S. cerevisia*e is a domesticated species that has continuously evolved in association with the production of alcoholic beverages (Mortimer, 2000; Martini, 1993; Naumov, 1996). Nowadays there are controversially arguments regarding the nature of *S. cerevisiae*. According to Davenport (1974), Rosini *et al*. (1982) and Sniegowski *et al*. (2002), isolation of *S. cerevisiae* from wild environment or agriculture products was uncommonly practiced. However Kurtzman and Fell (1998) reported that fruits, vegetables, drink and agriculture products are among important microhabitats for yeast species in the wild. Since Malaysia is recognized as one of the richest (megabiodiversity) flora and fauna in the world, attempt has been made to explore new indigenous *S. cerevisiae* strains. No work has been reported to look at the potential of indigenous *S. cerevisiae* as leavening agent bread making from local sources.

The leavening agents (yeasts) currently used by bakery industries in Malaysian are mostly imported from foreign countries such as Australia (Mauripan®), France (Saf-instant®), Canada (Fermipan®) and Turkey (Gold Pakmaya®). Thus, in this work the presence of yeasts in Malaysian local fruits and various plant parts is yet to be exploited. We isolated the *S. cerevisiae* strains from local fruits and some plant parts from several regions in Malaysia and characterized them to be used as leavening agent in bread making.

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Materials and Methods

Samples

Commercial *Saccharomyces cerevisiae* strain used in this study was ATCC no. 62418. A total of 30 samples of local fruits and various plant parts were collected from several states of Peninsular Malaysia included state of Selangor, Pahang, Kelantan and Terengganu. The collected samples were placed aseptically in sterile plastic bags and transferred in ice boxes (4°C) before brought to the laboratory until use. Selected fruits involved in this study were banana (*Musa acuminate*), papaya (*Carica papaya*), cocoa beans (*Teobroma cacao* L.), palm kernel pulp (*Cocos nucifera* L.), longan (*Dimocarpus longan* spp. *malesianus leenh*), soursop (*Annona muricata* L.), bamboo shoot (*Bambusa vulgaris*), snake fruit (*Salacca zalacca*), jackfruit (*Artocarpus heterophyllus*), duku Langsat (*Lansium domesticum*), honey, mango (*Mangifera indica*), durian (*Durio dulcis*); orange (*Citrus sinensis* L.); watermelon (*Citrulus vulgaris*), pineapple (*Ananas Comosus*), corn (*Zea mays*); rambutan (*Nephelium lappaceum*); manggosteen (*Garcinia mangostana*) ; sugar-cane juice (*Saccharum officinarum* L.) and coconut water (*Cocos nucifera*), fermented rice; nira nipah and samples of rice and soil. The samples then were subjected to the following procedures within 24–36 h after collection and transfer to the laboratory.

Enrichment procedures for yeast isolation

The enrichment procedure to isolate yeast species were carried out as method described by Thais *et al*. (2006). One ml (liquid) or 1 g (solid) was added into 50 ml of medium containing Grape Must (GM) and 27% (w/v) of sucrose with pH adjusted to 3.2. The medium was then incubated at 25 °C for 10 days. After 10 days incubation, 0.1 ml of liquid was inoculated onto Walerstein Laboratory (WL) nutrient agar (Oxoid) and incubated at 30°C for 3-5 days. Tentative yeasts isolated onto WL plates medium was then subcultured onto Yeast Peptone Dextrose (YPD) medium (10 g /L Bacto Yeast Extract, 10 g/L Bacto Peptone and 20 g/L glucose) (Oxoid, UK) which was added with chloramphenicol to avoid bacterial growth. The plates were incubated at 30°C for 3–8 days. The colonies were then counted and selected according to their morphological characteristics as described by Martini *et al*. (1996). Representative colony was picked from the plates and pure cultures were ready for identification procedures.

Microscope observation

A single colony of yeast was supplemented in a drop of sterile distilled water placed on glass slide and smeared until the smear dry off. The smear was then stained using diluted methylene blue dye, air dried and observed under light microscope at 100 x magnification.

Stress exclusion test

Stress exclusion test was conducted as described by Thais *et al*. (2006). The continuously growth of stress exclusion test was overall done for 15 days incubation onto different media. The ability to grow under different stress conditions were conducted by growing yeast isolates onto Yeast Peptone Glucose (YPG) (10 g/L yeast extract, 10 g/L peptone, 20 g/L glucose and 20 g/L agar) medium and incubated at 30ºC for 3 days. A single colony was then transferred and continuously grown on YPG medium and incubated at 37ºC for another 3 days, before further subculture the isolated yeast colony on YPG medium containing 8% (v/v) ethanol and incubated at 30ºC for 3 days. A single isolated colony on YPG with 8% ethanol was further subcultured on YPG supplemented with 20% (w/v) glucose and incubated under the same conditions as above. Finally the yeast cells were transferred on YP (10 g/L yeast extract, 10 g/L peptone) medium supplemented with 2% (w/v) sucrose and 8% (v/v) ethanol and incubated under the same conditions as above.

Ethanol tolerance test

The ability of the isolated yeast strains to grow in higher ethanol concentrations medium were tested by growing them in YPG broth containing 3 different concentration of ethanol, 10% , 13% and 15% (v/v), respectively and incubated at 30ºC for 72 hours (Thais *et al*., 2006).

Temperature tolerance test

The ability of the yeast to grow at higher temperatures was verified by plating the yeast isolates onto YPG medium and incubated at 4 different temperatures i.e. 25, 30, 37 and 45ºC for 72 hours (Thais *et al*., 2006).

Hydrogen sulfide production test

The ability of the yeast to produce hydrogen sulphide (H_2S) was examined by growing the yeast isolates on lead acetate medium (40 g/L glucose, 5 g/L yeast extract, 3 g/L peptone, 0.2 g/L ammonium sulfate, 1 g/L lead acetate and 20 g/L agar) and incubated 30ºC for 10 days (Ono *et al*., 1991).

Flocculation test

In this test, isolates were inoculated in 10 ml of YPG broth and incubated at 30ºC for 3 days. After incubation, tubes were agitated to observe the

flocculation formed (Thais *et al*., 2006).

Fermentative capacity test

In this test, the fermentative capacity ability media was prepared and the test was conducted as described by Atlas and Parks (1997). Prior before yeast cells grew into Yeast fermentation broth (YFB) (Peptone 7.5 g/L, yeast extract 4.5g/L; 1ml of 1.6% (w/v) bromothymo blue as an indicator), 6% (w/v) glucose, sucrose, fructose, maltose, lactose and galactose were autoclaved, separately. Only, raffinose was autoclaved at higher concentration of 12% (w/v). The yeast cells were grown at 30°C for 3 days. The YFB was added with respective sugar, then yeast cells were examined on the fermentative ability using different carbon source. The Durham tubes were also placed into the media to trap the carbon dioxide released. The fermented media were green in color and turn to yellow (acidic) or blue (alkaline) if the yeast cells have the ability to ferment the respective sugar.

Result and Discussion

Biochemical and physiological tests are important in order to confirm the suitability of the isolated yeast strains to be employed as baker's yeasts. A total of 30 different local fruits and plant parts were examined for the presence of indigenous *S. cerevisiae*. Since, the media used to isolate the yeast strains were selective for isolation of this species only 20 colonies of tentative yeasts have been isolated. The isolation of *S. cerevisiae* in the study was carried out using Grape Must (GM) broth and followed by subculturing in Walerstein Laboratory (WL) nutrient agar.

The 20 colonies yeast strains possess morphological features corresponded to *S. cerevisiae* species. Of the 20 yeast colonies, 18 isolates showed opaque, smooth, fluffy, regular colony and creamy in colour. While, yeast strain isolated from SMK9 and SDB10 showed similar characteristics except they were rough colonies compared to others and commercial *S. cerevisiae*. Similar finding was also observed by Kevin (2005) who reported that typical *S. cerevisiae* colonies were creamy and regular colony shape. Colony formed by cells of wild *S. cerevisiae* showed a fluffy morphology as reported by Kockova (1990), Cavalieri *et al*. (2001) and Kuthan *et al*. (2003). The morphological differences of yeasts colonies were very much depending on the absence of an extracellular matrix, the compactness of colony and the differences of cell shape within the colony (Kuthan *et al*., 2003).

The cells morphology of 16 yeast colonies observed under a light microscope with 100 times (100x) magnification showed an ellipsoid or ovoid shape which conformed the characteristic of *S. cerevisiae* (Greame, 2005). Fifteen yeast colonies have similar capability of budding where out of ten cells, 4-5 cells were budding. However, yeast strain of SMK9 (isolated from longan) showed extra capability of budding which out of ten, 7-8 cells were budding, indicated a higher growth rate when compared to other strains including the commercial yeast. With these characteristics shown, this strain should be able to perform a vigorous fermentation,

Table 1. Stress exclusion tests on isolated yeasts for temperature and cell osmotic pressure in high concentration of ethanol and sugar

Yeast Strains	Sources	Growth onto different media					Yeast strain potentially use as leavening agent
		YPG	Temperature 37°C	Ethanol (8%, v/v)	YPG (20%, v/v)	YPS (20%, w/v) + ETOH (8%, v/v)	
SN1	Pineapple	+++	+++	+++	+++	+++	Yes
SKS2	Oil palm tree	+++	+++	+++	+++	+++	Yes
SNR3	Nira nipah	+++	+++	+++	+++	+++	Yes
SB4	Papaya	+++	+++	+++	+++	+++	Yes
SM5	Mangosteen	+++	+++	+++	+++	+++	Yes
SD6	Duku	+++	+++	+++	+++	+++	Yes
STB7	Fermented rice	+++	+++	-	-	-	No
SO8	Honey orange	+++	+++	+++	+++	+++	Yes
SMK9	Longan	+++	+++	+++	+++	+++	Yes
SDB10	Soursop	+++	+++	+++	+++	+++	Yes
SRB11	Bamboo shoot	+++	+++	+++	+++	+++	Yes
SS12	Snake fruits	+++	+++	+++	+++	+++	Yes
SJ13	Sweet corn	+++	+++	+++	+++	+++	Yes
SK14	Cocoa	+++	+++	+++	+++	+++	Yes
SRT15	Rambutan	+++	+++	+++	+++	+++	Yes
SM16	Mango	+++	+++	+++	+++	+++	Yes
SC	Commercial strain	+++	+++	+++	+++	+++	Yes

Intensive response (+++); moderate response (++); low response (+); no response (-);
SC- Saccharomyces cerevisiae commercial as control
YPG – yeast peptone glucose medium
YPS – yeast peptone sucrose medium
Yes - potentially use as leavening agent
No – could not being use as leavening agent

Table 2. Effect of ethanol concentrations, temperatures, flocculation and hydrogen sulfide production of *Saccharomyces cerevisiae* strains

Yeast Strains	Ethanol Concentration (v/v)			Temperature °C				Flocculation	Hydrogen sulfide production	Potential to be employed as leavening agent
	10%	13%	15%	25	30	37	45			
SN1	+++	++	-	+++	+++	+++	+++	-	+	Yes
SKS2	+++	++	-	+++	+++	+++	+++	-	-	Yes
SNR3	+++	++	-	+++	+++	+++	+	-	+++	Yes
SB4	+++	++	-	+++	+++	+++	+	-	++	Yes
SM5	+	-	-	NT	NT	NT	NT	NT	NT	No
SD6	++	+	-	+++	+++	++	-	-	+	Yes
STB7	NT	NT	NT	NT	NT	NT	NT	NT	NT	No
SO8	++	+	-	+++	+++	++	-	-	+	Yes
SMK9	+++	++	-	+++	+++	++	-	+	++	Yes
SDB10	+++	++	-	+++	+++	++	-	+	++	Yes
SRB11	+++	+++	-	+++	+++	+++	-	-	+	Yes
SS12	+++	+++	-	+++	+++	+++	-	-	+	Yes
SJ13	+++	+++	-	+++	+++	+++	+++	-	+	Yes
SK14	+++	+++	+	+++	+++	+++	-	-	-	Yes
SRT15	+++	+	-	+++	+++	+++	-	-	+	Yes
SM16	+++	++	-	+++	+++	+++	-	-	+++	Yes
SC	+++	++	-	+++	+++	+++	-	-	+++	Yes

Intensive response (+++); moderate response (++); low response (+); no response (-);
SC- *Saccharomyces cerevisiae* commercial as control
NT – Not tested
Yes - potentially use as leavening agent
No – could not being use as leavening agent

Table 3. Fermentative capacity test

Yeasts strains	Carbon sources and carbon dioxide release in each sugar fermentation[a]							Yeast strains identified as *Saccharomyces cerevisiae*
	Glucose	Maltose	Fructose	Sucrose	Lactose	Galactose	Raffinose	
SN1	+	+	+	+	-	+	*+	Yes
SKS2	+	+	+	+	-	+	*+	Yes
SNR3	+	+	+	+	-	+	+	Yes
SB4	+	+	+	+	-	+	+	Yes
SM5	NT	NT	NT	NT	NT	NT	NT	No
SD6	+	+	+	+	-	+	+	Yes
STB7	NT	NT	NT	NT	NT	NT	NT	No
SO8	+	+	+	+	+	+	+	No
SMK9	+	+	+	+	-	+	+	Yes
SDB10	+	+	+	+	-	+	+	Yes
SRB11	+	+	+	+	-	+	+	Yes
SS12	+	+	+	+	-	+	+	Yes
SJ13	+	+	+	+	-	+	*+	Yes
SK14	+	+	+	+	-	+	*+	Yes
SRT15	+	+	+	+	-	+	*+	Yes
SM16	+	+	+	+	-	+	+	Yes
S. cerevisiae	+	+	+	+	-	+	+	Yes

[a]Assimilate carbon source and simultaneously release of carbon dioxide indicated as + ve; did not assimilate carbon source and not release carbon dioxide indicated as - ve.
Acidic fermentation (Broth turn into yellow)
*Alkaline fermentation (Broth turn into blue)
NT- not tested
Yes - potentially use as leavening agent
No – could not being use as leavening agent

according to Hough *et al*. (1971).

In stress exclusion test, 16 yeast colonies including commercial baker's yeast were examined in order to select the strains which were able to adapt in bread making conditions. Using strains which were not effectively adapted to stress condition could be a mistake due to impossible settlement during fermentation process (Thais *et al*., 2006). In all stress analyses, the yeast cells were initially grown on YPG medium to ensure yeast were in the similar state condition, followed by growing onto medium YPG incubated at 37°C, 8% (v/v) of ethanol, 20 % (w/v) of glucose and 20% (w/v) of sucrose with 8% (v/v) of ethanol. The yeast cells were grown continuously for 15 days to observe the cell viability due to each stress condition.

Strain survival of baker's yeast under various stress conditions could provide useful information on its ability to grow and carry out fermentation as impaired yeast. The impaired yeast growth (Ivorra *et al*., 1999) during the fermentation usually does not grow in optimal conditions and continuously exposed to several stress especially to osmotic and ethanol stress (Querol *et al*., 2003). In recent work, as indicated in Table 1, only yeast strain of STB7 was not able to fulfill those characteristics. While, the other 15 yeast strains including commercial yeast showed the necessary characteristics that suitable for bread making. Our findings were in agreement with Pataro *et al*. (2000) who reported that most of *S. cerevisiae* strains isolated from conventional fermentation processes were physiologically adapted to extreme conditions. In this case the strains were able to grow on medium (YP) containing 20% (w/v) glucose and 8% (v/v) ethanol and incubated at 37°C.

Since the yeast cells in bread making produce

ethanol as secondary metabolites the ethanol stress were conducted to observe its tolerance to ethanol. A suitable concentration of alcohol is needed in bread making in order to achieve the preferred flavor. As shown in Table 2, all yeast strains were able to grow in a medium containing 10% (v/v) of ethanol. At 13% (v/v) of ethanol concentration, only yeast strain SM5 (isolated from maggosteen) was not able to grow in the respective broth medium. Only yeast strain SK14 (isolated from cocoa) showed slow growth in 15% (v/v) of ethanol, while others including the commercial yeast strain failed to growth at respective concentration. High concentration of alcohol is reported to be toxic to the yeast by inhibiting the cells growth due to the destruction of the cell membrane (Ingram and Buttke, 1984; Leao and Uden, 1984). In this experiment, the highest concentration of ethanol that the commercial yeast strain was able to survive was at 13% (v/v). Those strains which were capable to grow in similar concentration were expected to have ability to produce similar quality of bread as commercial strain.

The selected yeast strains were also tested for its growth at high temperature (Table 2). Most of the yeast strains could tolerate to the temperature up to 37°C. Yeast strains of SN1 (isolated from pineapple), SKS2 (isolated from fruit oil palm) and SK14 (isolated from cocoa) were able to grow at higher temperature i.e. 45°C. There for it can be concluded that most wild yeasts isolated from different fruits and other plant materials studied were able to grow at high ethanol concentration (13%) and high temperature (37°C).

The flocculation abilities were also tested on the yeasts strains. According to Amri *et al*. (1982) and Miki *et al*. (1982), yeast cells which have ability to flocculate cause by cell adhesion process is an interesting characteristic in bread making and brewing industry. Results showed only strain SMK9 (isolated from longan) and SDB10 (isolated from soursop) had the ability to flocculate. The flocculation characteristic was determined by yeast cells sticking together and provides easy separation from the broth medium. This phenomenon has an economic effect on the production of yeast biomass due to it can reduce the energy cost biomass centrifuging (Iraj *et al*., 2002). In addition, flocculation properties of *S. cerevisiae* ensure a high cell density and large volume of harvested cells and also able to raise the ethanol productivity during the fermentation process (Kevin, 2005).

The production of hydrogen sulfide by 14 isolated *S. cerevisiae* strains was determined during fermentation of lead acetate medium (LA). Henschke and Lee (1994) reported that hydrogen sulfide production property was not useful for wine yeasts. Yeasts with elevate production of hydrogen sulfide are undesirable for bread making because confer flavor and taste that compromise the quality of the bread. However, in this study all yeast strains produced hydrogen sulfide including commercial yeast strain except strain SKS2 and S14 (Table 2). Since commercial strain showed high production of hydrogen sulfide (+++ intensive response), therefore other yeast strains were acceptable as bread making yeast.

In primary role of baker's yeast in dough development, fermentative capacity using carbon source simultaneously with the production of carbon dioxide is important parameter for bread making (Benitez *et al*., 1996). All yeast strains tested were identified as S. cerevisiae due to its ability to ferment sucrose, maltose, fructose, glucose, galactose and raffinose but not on lactose (Thais *et al*., 2006). Table 3 shows the fermentative ability of 14 S. cerevisiae strains including commercial strain using 7 different carbon sources. *S. cerevisiae* strains of SNR3, SMK9, SDB19, SRB11 and SS12 showed their ability to change the broth medium color from green to yellow (for sucrose, maltose, fructose, glucose and galactose) within 30-35 minute fermentation. But other strains took more than 24 hours incubation time to produce the similar results. This indicated that the former strains have gone through acidic fermentation and was able to initiate the fermentation process faster than the commercial strain. The commercial *S. cerevisiae* strain was acidic fermented yeast and the color changes took 18 hours of fermentation. In contrast, for carbon source of raffinose *S. cerevisiae* strains SN1, SKS2, SJ13, SK14 and SRT15 showed the changes of color from green to blue, which indicated an alkaline fermentation. All yeast strains including commercial strain did not ferment lactose except for strain SO8. Tarek (2001) reported the S. cerevisiae cells which were unable to ferment lactose were actually due to lack of lactase or β-galactosidase system. It was also showed that all yeast strains which utilized respective sugars also produced carbon dioxide (Table 3). The carbon dioxide released during dough fermentation process is prominent as a leavening agent of dough (Thais *et al*., 2006).

Conclusion

In overall experiments, it was noticed that from 13 yeast strains identified as *S. cerevisiae*, 5 yeast strains of SN3 (nira nipah), SMK9 (longan), SDB10 (durian belanda), SRB11 (bamboo shoot) and SS12 (salak or snake fruit) showed better fermentative

ability rather than *S. cerevisiae* commercial strains. It was evidence from this finding that the local fruits and plant parts could be sources of new *S. cerevisiae* strains which are potentially used as dough leavening agent for bread making.

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