Malays. Appl. Biol. (2018) 47(2): 83–88

ISOLATION AND IDENTIFICATION OF AMYLOLYTIC BACTERIA FROM RAGI

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Accepted 18 April 2018, Published online 25 May 2018

ABSTRACT

Ragi is a traditional dry starter culture frequently used in many fermented food products in Asian countries. Ragi contains starch that can be degraded by amylases provided by bacteria and other microorganisms during fermentation in producing fermented foods. This study was carried out to isolate bacteria from ragi and to screen for their ability to hydrolyse starch. Seven bacterial colonies were isolated from ragi, with three samples showing starch hydrolysis activity. Two of the starch-degrading bacterial isolates were identified using partial 16S rRNA gene sequencing as *Bacillus licheniformis* with 97% and 98% similarity, and one sample identified as a lactic acid bacteria, *Enterococcus faecium* (98%). Isolation and identification of these bacteria from ragi can provide a promising source of amylase that can be further studied and manipulated for the development of starter culture and to improve the quality of traditional ragi-based fermented food products.

Key words: Ragi, amylolytic activity, starch-degrading bacteria

INTRODUCTION

Ragi is one of the common starter culture used in Asian countries in producing different kinds of fermented foods. Ragi appears in several forms, and is typically made in the form of round and flattened dried cake. Some of the popular traditional foods produced from fermentation with ragi include *tapai* or *tapé* (a fermented glutinous rice or cassava), *tempe* (fermented soya bean) and *oncom* (fermented peanut press cake) (Owens, 2014). The names are varied at different regions, including ragi-*tapai* and ragi-*beras* in Malaysia, *sasad* in Sabah, ragi-*tapé* and ragi *peuyeum* (for cassava) in Indonesia, *jui-paing* or *look-pang* (grown on bran) in Thailand, *bubod levadura* in Philipines, *bakhar*, *murcha*, *ranu*, or *u-y-iat* in India (Steinkraus, 1995). The specific names following ragi indicates its particular uses such as ragi-*tempe* for soybean fermentation, ragi-*tapai* for *tapai* inoculant and ragi-*samsu* as an inoculum for Malaysian *samsu*-rice wine (Owens, 2014; Steinkraus, 1995).

A starter culture is defined as a preparation used in the production of a fermented food that contains a large number of cells of at least one microorganism to be added to a raw material as a means to accelerate and steering the fermentation process (Leroy & Vuyst, 2004). The metabolic activity of microorganisms in the starter culture contributes to the desired effects in the fermentation substrate, which is the food product (Vogel *et al.,* 2011). Hence, information of microbial diversity in a starter culture is essential to improve its quality and safety, for culture selection and nutritional enhancement (Oguntoyinbo & Narbad, 2012).

Microbial combination of yeasts and moulds isolated from Indonesian ragi have been found to produce a good quality *tapé* due to their high amylolytic activity (Hofrichter, 2010). In fact, amylase enzymes produced by some microorganisms in ragi can be beneficial for degrading starch available in the starter culture from substrates such as rice or cassava, which may contribute to the desired texture and flavour of the ragi-derived products.

Amylases are important hydrolase enzymes that can hydrolyse glycosidic linkages in starch and break them into polymers composed of glucose subunits (de Souza & de Oliveira, 2010). These starch-degrading enzymes are not only of great

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importance for food production, but also in various other industrial applications including pharmaceutical, textiles, detergent and paper industries. The application of amylases has also expanded in areas such as clinical, medicinal and analytical chemistry with the advances in biotechnology (de Souza & de Oliveira, 2010).

Amylases produced by microorganisms are more preferred compared to other sources due to the high rate of microbial production and can be produced in bulk, which makes them more cost effective (Sundaram & Murthy, 2014). Microorganisms are also easy to handle and can be engineered to obtain enzymes of desired characteristics (Naidu & Saranraj, 2014). Identification of bacteria and other microorganisms with the ability to produce starch-degrading enzymes is therefore important for their potential applications in food and biotechnological industries. This present study was carried out to isolate starch-degrading bacteria from ragi and to identify the bacteria using partial 16S rRNA gene amplification and sequencing.

MATERIALS AND METHODS

Isolation of Bacteria from Ragi

Three samples of commercial ragi were purchased from local market in Negeri Sembilan, Selangor and Perak. Four grams of each ragi were homogenized with 36 mL sterile NaCl solution (0.85%, w/v) to a homogenous suspension by using Stomacher 400 Circulator (Seward, England) for 1 minute and then a tenfold serial dilution in NaCl solution (0.85% w/v) was carried out to 10^{-4}. A volume of 0.1 ml of each dilution was transferred aseptically to De Man, Rogosa and Sharpe (MRS) agar (Oxoid) plates (62 g/L), and incubated at 37°C for 24 hours. Each of different character of bacterial isolates was further subcultured to obtain pure culture by streak plate method on MRS agar. The pure colonies were analysed under compound microscope (Olympus) with magnification of 1000X and oil immersion.

Screening of Bacteria for Amylolytic Activity

The microbial isolates were screened for amylolytic activity by starch hydrolysis test on commercial starch agar (Difco) (25g/L) supplemented with 5% (52g/L) MRS broth (Oxoid). The overnight MRS broth culture of bacterial isolates were streaked on the MRS-starch agar plate and incubated at 37°C for 48 hours anaerobically. After incubation, the plates were then flooded with Lugol's iodine (1% (w/v) potassium iodide and 0.1% (w/v) iodine). A clear zone of hydrolysis around the growth indicates positive result of starch hydrolysis, while the presence of blue colour around growth indicates negative result. The isolates that produce clear zones of hydrolysis were selected for further analysis.

Identification of Amylolytic Bacterial Isolates

Genomic DNA was isolated from the pure culture of the amylolytic bacteria using the Wizard Genomic DNA Purification kit (Promega, USA) according to the manufacturer's guidelines. *Lactobacillus plantarum* ATCC 8014 (American Type Culture Collection) was used as positive control. Quantification of DNA was carried out using nano-spectrophotometer (Implen, Germany). PCR amplification of the 16S rRNA gene of bacterial isolates was performed according to the method by Emerenini *et al.* (2011) with minimal changes. Universal primers that were used to amplify the 16S rRNA gene are 27F: 5'-AGA GTT TGA TCC TGG CTC AG-3' and 1492R: 5'-ACG GCT ACC TTG TTA CGA CTT-3' (Integrated DNA Technologies). The PCR reaction mixture consist of 10μL 2x Taq Master Mix (Bioron), 1μL of each primer (2.5 μM), 6.5μL nuclease free water and 1.5μL template DNA. The thermocycler (Eppendorf Mastercycler Gradient) was set as follows: initial denaturation at 94°C for 1 minute, denaturation at 95°C for 30 seconds, annealing at 44°C for 30 seconds and extension at 72°C for 2 minutes in 30 cycles.

PCR products were analysed using 1.0% (w/v) Agarose gel D-1 Low EED (Pronadisa, Spain) stained with ethidium bromide in 1xTAE buffer (Bio Basic, Canada). The DNA bands were visualized under UV trans-illuminator (BIORAD), and the sizes of DNA fragments were estimated using a standard 1 kb DNA ladder (Norgen Biotek Corporation, Canada). The PCR amplified products were sequenced at the First BASE Laboratories Sdn Bhd (Selangor). The partial 16S rRNA sequences obtained were read using Chromas Lite software and analysed using Basic Local Alignment Search Tool (BLAST) system of National Center for Biotechnology Information (NCBI) to determine sequence similarities and establish the isolate identity percentage compared to the existing sequences in the GenBank database.

RESULTS AND DISCUSSION

Seven colonies isolated from ragi showed the morphological characteristics of bacteria after 24 hours incubation on MRS agar media (Table 1). The samples were named JA2, KA4, LA4, OB3, PB4, QC2, RC2, with the middle alphabet representing ragi samples collected from different locations; A from Perak, B from Selangor and C from Negeri Sembilan. The six bacterial isolates were subsequently screened for amylolytic activity on starch agar medium, with MRS broth as the negative control. Three bacterial isolates KA4, OB3 and PB4

Table 1. Microscopic observation and starch hydrolysis of bacterial isolates

Samples	Cell Morphology	Starch Hydrolysis
JA2	Cocci	Negative
KA4	Rod	Positive
LA2	Cocci	Negative
OB3	Rod	Positive
PB4	Rod	Positive
QC2	Cocci	Negative
RC2	Cocci	Negative

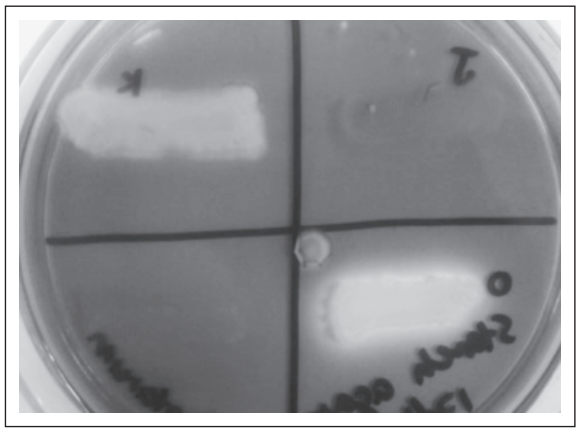

Fig. 1. Halo zone formation of isolate KA4 (top left) and OB3 (below right).

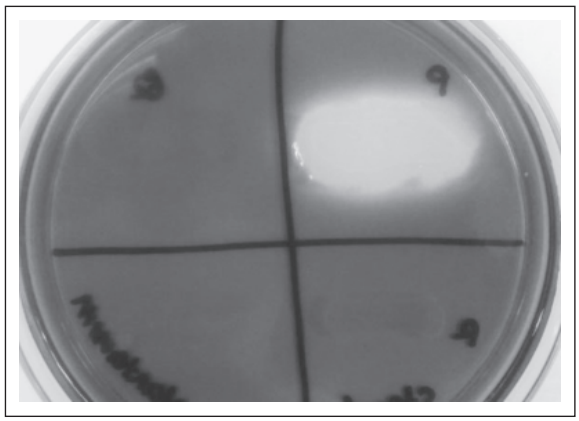

Fig. 2. Halo zone formation of isolate PB4 (top right).

showed starch hydrolysis activity based on the formation of halo zone when flooded with Lugol's iodine solution (Figure 1 and 2). Starch in the presence of iodine gave a bluish colour to the medium, indicating the absence of amylolytic activity (negative result) as observed on isolates JA2, LA2, QC2 and RC2. Starch that was hydrolysed formed a clear zone of hydrolysis surrounding the growth of the bacteria (positive result) as observed on isolates KA4, OB3 and PB4.

Amplification and sequencing of partial 16S rRNA gene were done to identify the three bacterial isolates that showed the ability to hydrolyse starch, KA4, OB3 and PB4. PCR amplification of the partial 16S rRNA gene was carried out using the universal 27F and 1492R primers. The PCR products of *Lactobacillus plantarum* (positive control), KA4, OB3 (Figure 3) and PB4 (Figure 4) showed DNA bands in the range of approximately 1000 to 1500 bp in size. The partial 16S rRNA sequence comparison against the BLAST (NCBI) database entries revealed that isolate KA4 has 96% similarity to *Enterococcus faecium,* while isolates OB3 and PB4 have 97% and 98% similarities to *Bacillus licheniformis* respectively (Table 2).

The two starch-degrading bacterial species isolated from commercial ragi in the present study have been commonly associated with various types of starter culture and fermented foods in previous researches. *Enterococcus faecium* is a lactic acid bacteria (LAB) that was previously found in Indonesian ragi *tapé*, Vietnamese alcohol fermentation starter culture *bahn men*, the traditional Turkish fermented milk *boza* and Taiwanese black bean soy sauce *inyu* (Sujaya *et al.,* 2001; Thanh *et al.,* 2008; Kivanc *et al.,* 2011; Wei *et al.,* 2013). *Enterococcus* spp. is also among the most dominant LAB found in Indonesian ragi *tapé*, which may contribute to the flavour of the ragi-based product such as *tapé* and *brem* (Sujaya *et al.,* 2010). Different strains of *Enterococcus faecium* used in Greek feta cheese starter culture have also shown positive effects on the overall sensory profile of the full-ripened cheeses as well as the taste, aroma, colour and structure (Sarantinopoulos *et al*., 2002), indicating the potential of this species in improving food quality.

The bacteria of *Bacillus* spp. have also been linked to fermentation processes in various fermented foods such as *thua nao*, cocoa and *kinema* (Inatsu *et al.,* 2006; Ouattara *et al.,* 2008; Tamang, 2015). *Bacillus licheniformis* was found to be predominant in *Daqu*, a traditional fermentation starter used to produce attractive flavoured foods such as vinegar, soy sauce and Chinese liquor (Yan *et al.,* 2013). Certain strains of *B. licheniformis* isolated from *Daqu* were also shown to exhibit antimicrobial activities, which may explain their dominance distribution (Yan *et al.,* 2013). A number of *B. licheniformis* strains have also been established to be salt tolerant and showed significant ability to produce some of the major industrially important extracellular hydrolytic enzymes that include α-amylase, glucoamylase, protease, pectinase and cellulase (Ghani *et al.,* 2013). Besides, a *B. licheniformis* strain was also found to produce thermostable α-amylase making it potentially suitable in many industries such as

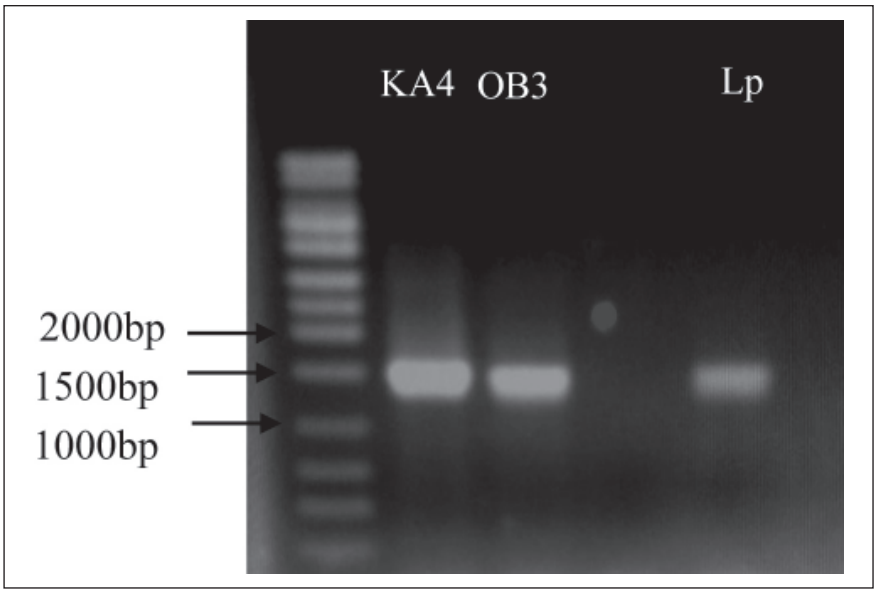

Fig. 3. PCR products of 16S rRNA gene of samples KA4, OB3 and *Lactobacillus plantarum* (Lp) as the control, with 100bp molecular weight marker.

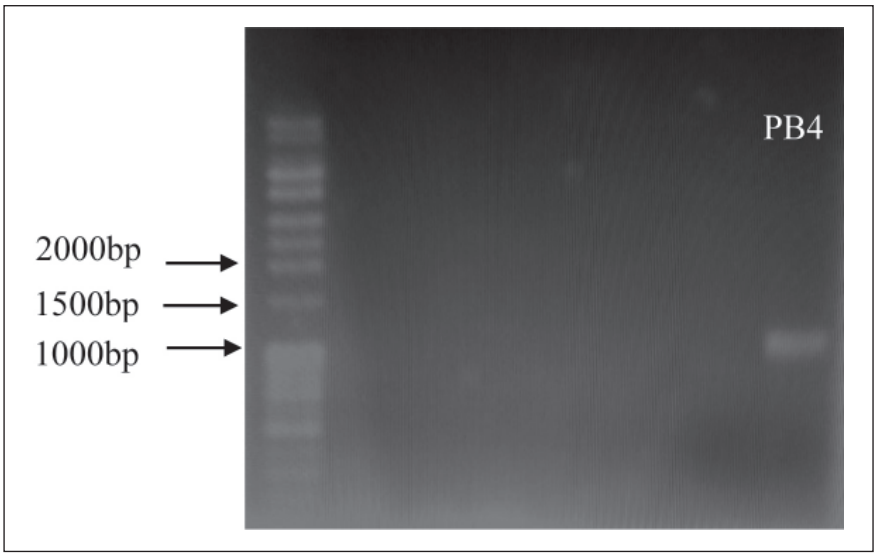

Fig. 4. PCR products with molecular weight marker (1kb) and PCR amplified 16S rRNA gene of sample PB4.

Table 2. Sequencing analyses of partial 16S rRNA gene of starch-degrading bacterial isolates

Samples	Closest relatives in GenBank	% Identity	Accession number*
KA4	*Enterococcus faecium*	96	KJ026644.1
OB3	*Bacillus licheniformis*	97	JQ045283.1
PB4	*Bacillus licheniformis*	98	KJ526872.1

*Corresponding accession number in the GenBank database.

those involving gelatinization and liquefaction to economize the processes (Vaseekaran *et al.,* 2011).

Further investigation of the isolated bacterial strains from this study are needed to determine their safety and stability in the development of ragi starter culture as well as the probiotic potential of the *Enterococcus faecium* strain. These bacteria may also be manipulated in order to improve the quality and acceptability of traditional fermented foods, and for enhancement of their texture and organoleptic structure.

CONCLUSION

Three samples of bacterial colonies isolated from commercial ragi starter culture were shown to have starch-degrading enzyme activity which may contribute to the organoleptic properties of ragi-derived products. The bacterial colonies capable of hydrolysing starch were identified through partial sequencing of the 16S rRNA gene, revealing a lactic acid bacteria *Enterococcus faecium* and two samples of *Bacillus licheniformis*. Further characterisation of the bacterial species can be useful for their potential in development of starter cultures with improved quality and safety. Starter culture development is important for the potential of commercial production and marketing of ragi-based foods. The starch-hydrolysing bacteria can also be further characterised for their potential as the source of amylase that may be a great value for industrial applications.

ACKNOWLEDGEMENTS

The authors acknowledged all members of Food Biotechnology program, Faculty of Science and Technology (FST), Universiti Sains Islam Malaysia (USIM), Nilai, Negeri Sembilan. We are grateful to all reviewers of this paper. This research was funded by the USIM Short Term Grant – Ref: PPP/USG-0114/FST/30/10414.

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