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Isolation and identification of lactic acid bacteria from Indonesian fermented foods as γ-aminobutyric acid-producing bacteria

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Abstract

γ-aminobutyric acid is the most abundant inhibitory neurotransmitter in brain and has various physiological functions. The aim of this study was to isolated and screened GABA-producing lactic acid bacteria originally from Indonesia. Twelve fermented foods were considered in this study and the ability of LAB as GABA-producing bacteria were analyzed using TLC cellulose plates and pre-staining chromatography method. Six isolates (IFK-10, IFK-11, IFK-12, FN-12, FN-14, FN-15) were able to convert MSG to GABA during 24 h of cultivation. Two strains IFK-10 and IFK-11 showed the highest amount of GABA concentration i.e 2.68 and 2.06 mg/ml, respectively. These strains were identified as *Lactobacillus plantarum* IFK-10 and *Pediococcus pentosaceus* IFK-11. Two strains of LAB from Indonesian as GABA-producing bacteria have a promising prospect and could support the development of functional foods.

Introduction

γ-Aminobutyric acid (GABA) is the most abundant inhibitory neurotransmitter in brain and widely distributed in plants, animals and microorganisms (Brambilla *et al.*, 2013). GABA has various physiological functions such as antidepressant (Chih *et al.*, 2013), induction of hypotension (Inoue *et al.*, 2003), and cholesterol lowering effect (Watanabe *et al.*, 2012). GABA is also could prevent obesity by ameliorating oxidative stress in high-fat diet in mice (Xie *et al.*, 2014) and effectively prevent diabetic condition by strongly secretagogue insulin from pancreas (Hagiwara *et al.,* 2004; Adeghate and Ponery, 2012). A study by Inoue *et al*. (2003) and Mathieu-Pouliot *et al*. (2013) reported that GABA-enriched dairy product was significantly decrease systolic blood pressure in men and mildly hypertensive men.

GABA is primarily formed by glutamic acid decarboxylase enzyme (GAD, EC 4.1.1.15) which catalyzes the irreversible α-decarboxylation of glutamic acid to produce GABA (Li and Chao, 2010). Many microorganisms can produced GAD such as bacteria (Komatsuzaki *et al.*, 2005), fungi (Kono and Himeno, 2000) and yeast (Masuda *et al*., 2008). Several strains of lactic acid bacteria (LAB) have an ability to produce GAD and its biochemical properties have been characterized (Komatsuzaki e*t al*., 2005; Siragusa *et al*., 2007; Komatsuzaki *et al*., 2008).

Due to physiological effect of GABA, many studies have been pursued and recent research undertaken to increase GABA content in food since direct addition of GABA to food is considered unnatural (Kim *et al*., 2009; Villegas *et al*., 2016). Therefore, it is necessary to produce and increase GABA naturally in food. Various LAB have been reported as GABA- producing bacteria. LAB are the most important microorganisms and play important role in food fermentation. LAB generally regarded as safe (GRAS) and have been extensively studied their role in food industries, bioactive compound producing-bacteria, dairy industries and as probiotic. Most research on GABA production has focused on the production of microbial rather than chemical synthesis due to corrosive nature of the reactant compound (Diana *et al.*, 2014). Several LAB strains isolated from food source have been shown to have an ability to produce GABA including *L. namurensis* NH_2 and *P. Pentosaceus* NH8 from Nham (Ratanaburee *et al*., 2013), *L. paracasei* NFRI 7415 from Japanese fermented fish (Komatsuzaki *et al*., 2005), *L.paracasei* PF6, *Lactococcus lactis*

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PU1 and *L. brevis* PM17 from cheese (Siragusa *et al.*, 2007), *L. brevis* 119-2 and *L. brevis* 119-6 Tsuda Kabu (Watanabe *et al.*, 2012).

Indonesia has many kinds of fermented foods which are fermented spontaneously by indigenous LAB and other microorganisms such as yeast and molds. Some species of LAB have been isolated from dadih, tempoyak, salted cabbage, gatot, growol etc. A previous study (Rahayu *et al.*, 2015) reported that LAB isolated from Indonesian fermented foods could potentially be probiotics as they are able to survive in acidic conditions, resistance to high bile salts and has an antibacterial activity. However, there is a little information about LAB from fermented foods Indonesian origin as GABA-producing bacteria. In this study LAB were isolated from Indonesian fermented foods and the ability to produce GABA were screened. A new strain of LAB from Indonesian could support the development of functional foods fortified with GABA.

Materials and Methods

Materials

Twelve fermented foods were considered in this study, consisting of two kinds of fermented cassava (namely growol and tape ubi), fermented soy beans, tempeh, salted cabbage, salted fruits, chicken sausage, beef sausage, and bekasam (fermented fish with rice). These samples will be used for selecting GABA-producing LAB. Medium of de Man Rogosa agar was from Oxoid. GABA standard were purchased from Sigma Aldrich. All chemicals reagent used in this study were analytical grade.

Isolation and screening GABA-producing LAB by thin layer chromatography

One mg of samples were serially diluted and inoculated on MRS agar containing 1% calcium carbonate ($CaCO_3$) (Oxoid) then incubated at 37^0C for 24 h under aerobic conditions. The isolates which formed clear zone were considered as LAB. Single colonies were streaks onto MRS agar to obtain pure cultures. In order to select LAB with high GABA-producing ability, bacteria were grown on MRS supplemented with MSG 5% and incubated at 37^0C for 24 h, and then the cultured broth was centrifuged at 1000 rpm for 10 min. The resulting supernatants were filtered using membrane filter.

A 2 µl of supernatants were spotted on TLC silica plates activated using butanol: acetic acid: aquades (5:3:2) containing 0.4% ninhidryn (Qiu *et al.*,2010). After development, the plate was directly dried at 90^oC for 5 min. The GABA-producing strain were identified based on gram staining, observation under microscope and by 16S rDNA sequence determination.

Quantification of GABA-producing bacteria

GABA concentrations in cultured broths were determined by pre-staining paper chromatography (Li *et al.,* 2009). A 2 µl of supernatants were spotted onto cellulose plates and developed at 30^0C with n-butanol-acetic acid-water (5:3:2) containing 1.2% ninhydrin. After development, GABA spots were scratched out from the paper and were extracted with 5 ml of 75% alcohol (v/v):0.6% cupric sulfate (w/v) (38:2) at 40^0C. The absorbance was read using spectrophotometer at 512 nm. The purity of GABA was also determined by HPLC as reference procedures.

Identification of GABA-producing bacteria

Identification was based on 16S RNA according to Ivanova *et al.* (2008). Genomic DNA was isolated using Kit Isoplant II (isoplant code No. 310-04151, Nippon Gene, Toyama, Japan). A 1 µl of supernatant was used as a template in PCR. Amplification was conducted with infinigen thermocycler machine. The 16S rRNA genes were amplified using a pair of universal primers corresponds to positions 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3').The amplification procedures were as follows: initial denaturation at 94^0C for 5 min, followed by 40 cycles at 94^0C for 20 sec, the annealing temperature at 40^0C for 2 min and the elongation temperature at 72^0C for 30 sec, and additional final elongation temperature at 72^0C for 5 min. A 5 µl PCR product were analysed using gel electrophoresis. The partial 16S rDNA sequence was compared with the GenBank database in the NCBI using BLAST and phylogenetic tree was performed using MEGA 4.

Results and Discussion

Screening and identification of GABA-producing LAB

GABA-producing LAB were isolated from fermented foods and isolated strains which formed a clear zone on MRS plates containing $CaCO_3$ at 37^0C for 24 h, were considered as LAB. Six isolates (IFK-10, IFK-11, IFK-12, FN-12, FN-14, FN-15) originally from fermented soy beans and fermented fish showed high GABA production by TLC (Figure 1). The isolates were able to convert MSG during 24 h of incubation and showed the same Rf as that of GABA standard (Rf = 0.61). Two isolates IFK-10

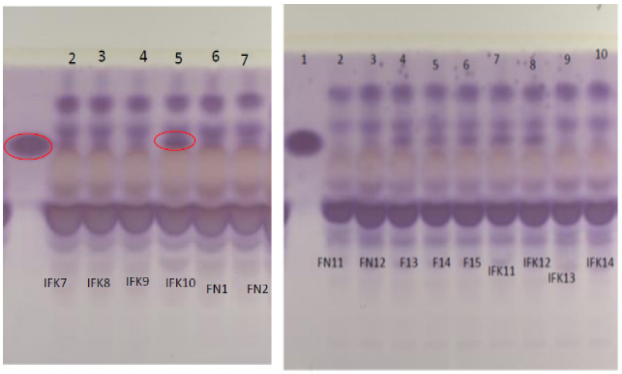

Figure 1. Screening of GABA producing bacteria using TLC cellulose plate. Plate A, lane 1. GABA standard, lane 2.supernatant of IFK-7, lane 3. Supernatant of IFK-8, 4. Supernatant of IFK-9, 5. Supernatant of IFK-10, lane 6. Supernatant of FN-1 and lane 7. Supernatant of FN-2. Plate B, lane 1, GABA standard, lane 2. Supernatant of FN-11, lane 3. Supernatant of FN-12, lane 4. Supernatant of F-13, lane 5. Supernatant of F-14, lane 6, Supernatant of F-15, lane 7. Supernatant of IFK-11, lane 8. Supernatant of IFK-12, lane 9. Supernatant of IFK-13 and lane 10. Supernatant of IFK-14. All isolates were grown in MRSB supplemented with 5% of MSG and incubated for 24 h at 37^{0}C.

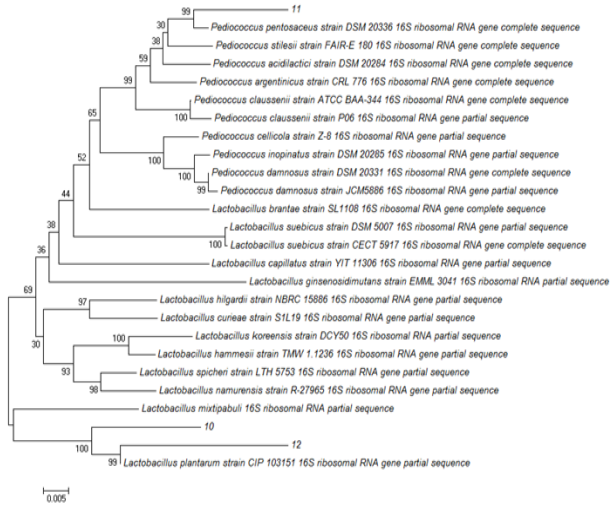

Figure 2. phylogenetic tree of lactic acid bacteria

and IFK-11 showed high surface area determined by TLC scanner. The surface area of strains IFK-10 and IFK-11 have reached to 66,712.2 and 30,435.7. These results showed that strains IFK-10 and IFK-11 could convert monosodium glutame after 24 hours of incubation. The amounts of GABA in supernatant were further analyzed by pre-staining chromatography and the purity of this product was determined with HPLC.

Two isolates produced the highest amount of GABA were Gram positive, short rod and cocci cell type. The isolates were identified as *Lactobacillus plantarum* and *Pediococcus pentosaceus* (IFK-10 and IFK-11, respectively) according to phylogenetic tree (Figure 2). The partial complete sequence (1492 bp) of the 16S rRNA gene of strain IFK-10 and IFK-11 were amplified by PCR. The results were indicated that isolates IFK-10 and IFK-11 exhibited high similarity value (99%) to *Lactobacillus plantarum* CIP 103151 and *Pediococcus pentosaceus* DSM 20336. The result of phenotypic characters and phylogenetic analysis clearly indicated that strain IFK-10 and IFK-11 belonging to *L. plantarum* and *P. pentosaceus,* respectively. These results were in agreement with Siragusa *et al.,* (2007) and Ratanaburee *et al*., (2013) reported that strains *L. plantarum* C48 and *P. pentosaceus* NH8 isolated from Italian cheese and Nham were considered as a high GABA – producing lactic acid bacteria

Table 1. GABA content of some strains as measured by pre-staining chromatography. The absorbance was read at 512 nm.

Isolates	Rf	surface area (AU)	GABA (mg/ml)
IFK 10	0.61	66712.2	2.68
IFK 11	0.61	30435.7	2.06
IFK 12	0.6	28790.7	1.11
FN 13	0.61	25618.8	1.37
FN 14	0.61	14352.2	1.72
FN 15	0.61	28995.9	1.12

Several strains or species of LAB have been reported as GABA - producing bacteria and the ability to convert MSG to GABA is a strain dependent. Almost all strains or species of GABA – producing bacteria were isolated from traditional fermented foods such Nham (Ratanaburee *et al.*, 2013), Italian cheese (Siragusa *et al*., 2007), and paocai (Li *et al.*, 2008). Moreover, all isolation sources are rich in glutame which is an essential source to screen GABA – producing bacteria. Almost all isolates reported are belongs to lactobacilli species. This is our first report strains *L. plantarum* IFK-10 and *P. pentosaceus* IFK-11 from fermented soy beans as an isolation source.

Quantification of GABA

The amount of GABA in supernatant was determined using pre-staining chromatography method. Pre-staining chromatography are suitable for detection of GABA and the method is more clean, simple, convenient, inexpensive and reproducible (Li and Chao, 2010). This method has almost the same Rf values to those of traditional method. Two strains IFK-10 and IFK-11 produced high concentration of GABA in supernatant during 24 h of incubation and reached to 2.68 and 2.06 mg/ml in MRSB containing 5% of MSG compare to other four strains (Table 1). Thanh-Binh *et al*. (2014) reported that *L. brevis* isolated from kimchi was able to produce GABA up to 44.4 g/l after 72 h of incubation in MRSB containing

6% of MSG. Several studies have reported that LAB isolated from fermented foods is able to convert MSG as substrate to GABA.

High content of MSG are required to improve the production of GABA by LAB. Recently, *L. brevis* CRL 1942 isolated from Real Hornillos quinoa sourdough was able to convert 270 mM of MSG to GABA reached to 255 mM after 48 h of cultivation at 300C (Villegas *et al.*, 2016). Siragusa *et al.* (2007) reported that strain *Lactobacillus brevis* PM17, *Lactobacillus plantarum* C48, *Lactobacillus paracasei* PF6, *Lactobacillus delbrueckii* subsp. *bulgaricus* PR1 and *Lactococcus lactis* PU1 isolated from 22 Italian cheese varieties produce GABA up to 15-63 mg/kg in different culture media. In addition, *L. brevis* CECT8183 isolated from artisan Spanish cheese produced 100 mg/l of GABA (Diana *et al.*, 2014). LAB with high GABA production is related with GAD enzyme activity. The concentration of glutamic acid in food matrix should be high enough to increase GABA production. In this study suggest that almost all isolates may exhibit different GABA – producing ability depending on glutamate/GABA content in the samples. GABA-producing bacteria could exhibit high production of GABA in samples with high glutamate/GABA content than those from the samples with low glutamate/GABA content. In addition, Siragusa *et al.* (2007) reported that only four isolates of LAB such as *L. paracasei* PF6, *L. bulgaricus* PR1, *L. lactis* PU1 and *L. brevis* PM17 isolated from different variety of cheese with the highest GABA production and exhibit the highest level of GABA.

Conclusion

In conclusion, six isolate of LAB from fermented soy beans have the ability to convert MSG to GABA during 24 h of cultivation. Two strains IFK-10 and IFK-11 showed the highest amount of GABA concentration during 24 h of cultivation i.e 2.68 and 2.06 mg/ml, respectively. These strains were identified as *Lactobacillus plantarum* (IFK-10) and *Pediococcus pentosaceus* (IFK-11), short rod shapes, cocci cell type and gram positive bacteria. In this study we obtained two novel LAB strains from fermented soy bean as GABA-producing bacteria hence could be used as starter culture that has a functional trait to develop functional foods.

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