Screening of glutaminase-producing bacteria from Kung-Som

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Abstract

Glutaminase catalyzes the hydrolytic degradation of *L*-glutamine to *L*-glutamic acid and ammonia. The screening of both glutaminase-producing bacteria and lactic acid bacteria (LAB) from Kung-Som was carried out. Among 360 strains, only 9 strains of coagulase-negative staphylococci (CNS) bacteria and 10 strains of LAB exhibited the glutaminase activity after cultivation on nutrient broth and MRS broth, respectively for 24 h at 37 ºC. CNS bacterial strain J03SM3 and LAB strain J01SM17 showed the highest glutaminase activity at 0.328 U/ml and 0.145 U/ml at pH 7.0, respectively. The 16S rDNA sequencing analysis of strain J03SM3 and J01SM17 showed that they were closely related to *Staphylococcus piscifermentans* and *Lactobacillus rhamnosus*, respectively.

Keywords: *L*-glutamine, glutaminase, bacteria, lactic acid bacteria, Kung-Som

Introduction

Kung-Som is a traditional fermented shrimp widely distributed in the south of Thailand. It is made from shrimp, sugar, salt and water and fermented with the natural microbial flora. The main microorganisms found in fermented shrimp are lactic acid bacteria (LAB) and coagulase-negative staphylococci (CNS) (Hajar *et al*., 2013). Most customers prefer choosing Kung-Som by its taste that obtained from the glutaminase activity by bacteria. These enzymes convert glutamine in food products to glutamic acid (Weingand-Ziadé *et al*., 2003; Liu *et al*., 2011).

Glutaminase (EC 3.5.1.2) is an enzyme that catalyzes the hydrolysis of L-glutamine to L-glutamic acid and is widely distributed in plants, animal tissues and microorganisms including bacteria, yeast and fungi (Kashyap *et al*., 2002; Jeon *et al*., 2009). L-glutamic acid (monosodium glutamate) is a high flavor amino acid in foodstuffs (Jeon *et al*., 2009) which is an important for umami taste factor. In addition, it plays an important role in the production of fermented foods by enhancing the umami taste. Accordingly, the hydrolysis of glutamine is a key process in the food industry in order to enhance the umami taste. In addition, glutaminase is one of the key enzymes responsible for increasing the glutamate concentration in soy sauce fermentation (Weingand-Ziadé *et al*., 2003) and other fermented foods such as sourdough, cheese and Nham (Thongsanit *et al*., 2009). Microbial glutaminases have received significant attention in the food industry owing to its potential as a flavor modulating agent. It increases the glutamic acid content of the food imparting savory flavor (Thongsanit *et al*., 2009). In traditional Japanese soy sauce fermentation, the glutaminase produced by koji molds (*Aspergillus oryzae* or *A. sojae*) plays a key role in the release of glutamic acid (Weingand-Ziadé *et al*., 2003). The objectives of this work were to screen the glutaminase-producing bacteria from Kung-Som and select the most suitable strains for using as starting culture in order to improve the quality of Kung-Som production.

Methodology

Isolation of lactic acid bacteria (LAB) and coagulase-negative staphylococci (CNS)
Samples (25 g) were added to 225 mL of sterile 0.05 mM potassium phosphate buffer pH 7.2, containing 10 mg/mL NaCl (PBS) and shaken for 5 min (Hwanhlem *et al*., 2010). Appropriate decimal dilutions were prepared in PBS buffer and poured into sterile petri dish on M17 agar (Merck, Germany), de Man Rogosa and Sharpe (MRS; Labscan Asia Co., Ltd, Bangkok, Thailand) agar containing 0.004% bromocresol purple and mannitol salt agar (MSA; Merck, Germany), for separating LAB and CNS, respectively, and incubated at 37ºC for 24 h. Growing colonies were individually picked and streaked on M17 agar, MRS agar containing 0.004% bromocresol purple for LAB and Nutrient agar (NA) for CNS bacteria.

This procedure were repeated 2-3 times in order to purify the strains.
Each of the strain was tested for catalase activity by placing a drop of 3% hydrogen peroxide solution on the cells. Immediate formation of bubbles indicated the presence of catalase in the cells. All strains were Gram-stained and only the isolates which catalase-negative and Gram-positive were maintained in MRS broth and M17 broth containing 25% glycerol at -20 ºC. The strains which catalase positive and Gram-positive were tested for coagulase activity using plasma. Only the strains presenting catalase-positive, Gram-positive and coagulase-negative were maintained on NB broth containing 25% glycerol at -20 ºC. For routine analysis, strains were sub-cultured twice in MRS broth or M17 broth for LAB and NB for CNS bacteria for 24 h at 37 ºC.

Primary screening of glutaminase-producing strain
The glutamine medium (G-medium) was used for the isolation of glutaminase-producing bacteria. G-medium is containing 1% (w/v) glutamine (Merck, Germany) as a sole carbon and 0.05% (w/v) yeast extract as a nitrogen source, 0.1% (w/v) K_2HPO_4, 0.1% (w/v) KH_2PO_4, 0.01% (w/v) $MgSO_4.7H_2O$, 0.1% (w/v) NaCl and 0.0015% (w/v) phenol red used as indicator (pH 7.0) (Wakayama *et al*., 2005). The modified MRS was used for the isolation of glutaminase-producing LAB which containing 1 % (w/v) glutamine and 0.5% (w/v) D-glucose as carbon source, 1% (w/v) polypeptone, 1% (w/v) beef extract, 0.5% (w/v) yeast extract, 0.1% (w/v) tween 80, 0.2% (w/v) ammonium citrate, 0.5% (w/v) CH_3COONa, 0.01% (w/v) $MgSO_4{\cdot}7H_2O$, 0.005% (w/v) $MnS0_4.H_2O$, 0.2% (w/v) K_2HPO_4 and 0.0015% (w/v) phenol red as indicator (pH 7.0). The selected CNS bacteria and LAB were spotted on G-medium agar plates and modified MRS agar plates, respectively. After incubation at 37 ºC for 24 h, the growing colonies exhibiting pink zone around colony were confirmed the glutaminase-producing strains with Thin layer chromatography (TLC). The fermented glutamine medium was centrifuged (10,000×g, 4 °C, 10 min). Two µl of cell culture supernatant of selected strains were subjected to TLC for the confirmation of glutaminase production. Solvent mixture of n-butanol, acetic acid and water (5:3:2 v/v) was used as a mobile phase. The developed chromatogram was dried and sprayed with 0.5% (w/v) ninhydrin and heated at 110 ºC for 4 min. Spot of the same R_f value with the 1% (w/v) standard glutamic acid (Sigma, St Louis, USA) were compared for intensity (Qiu *et al*., 2010).

Determination of L-glutaminase activity
L-glutaminase activity was determined using L-glutamine as substrate and the product was ammonia which measured by Nessler's reagent (Sigma, St Louis, USA) according to method (Sabu *et al*., 2000). One unit of L-glutaminase activity was defined as the amount of enzyme

that liberated 1 µmol of ammonia under optimal assay conditions. Assays were done in triplicate and the mean enzyme activity was expressed as U/ml.

Identification of glutaminase-producing strain

The 16S rDNA genes sequencing were amplified by polymerase chain reaction (PCR).The cell suspension of LAB and CNS bacteria obtained upon growth in MRS broth and NB, were centrifuged (10,000×g, 4 °C, 10 min) to collect cell pellet. The cell pellet was washed with TE buffer (pH 8.0), centrifuged and the supernatant was discarded. Cells were re-suspended in 200 µL of TE buffer and DNA was extracted with genomic DNA minikits (Genaid, Taiwan). Primers used for PCR were 8F (5′-AGAGTTT-GATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Reysenbach *et al*., 2000). The PCR conditions consisted of an initial denaturation at 95 ºC for 5 min, 30 cycles of 95 ºC for 1 min, 55 ºC for 0.5 min, and 72 ºC for 1 min. The amplified PCR products were analyzed by 1.5% agarose gel electrophoresis with a 500-bp DNA ladder marker as the molecular weight standard. The PCR product was purified with Gel/PCR DNA Fragments Extraction Kit (Genaid, Taiwan) and the DNA sequence was determined using the 8F and 1492R primers by DNA sequencer. The non-aligned sequence was submitted to the GenBank database. (http://www.ncbi.nlm.gov/)

Results and Discussion

Isolation of lactic acid bacteria (LAB) and coagulase-negative staphylococci (CNS)

The isolation of LAB and CNS bacteria from Kung-Som were carried out. The MRS, M17 agar and MSA were used as a preliminary screening medium for rod, cocci and CNS, respectively. As shown in Table 1, 360 strains grew on MRS, M17 and MSA plates. However, only 167 strains were identified as LAB and 116 strains were classified as CNS. In addition, 96 strains from MRS medium, 76 strains were rod and 20 strains were cocci. In generally, MRS medium is composed of magnesium and manganese salts supply the nutritive elements required for the growth of lactobacilli. Among 71 strains from M17 medium, 12 strains were rod and 59 strains were cocci. M17 medium contained yeast extract supplies B-complex vitamins which stimulate the growth of cocci bacteria, disodium-β-glycerophosphate buffers stabilizes the pH during bacteria growth and ascorbic acid were stimulated growth of cocci. One-hundred and sixteen isolates of staphylococci (CNS) were obtained from MSA which contained high concentrations of sodium chloride, the sodium chloride inhibited most bacteria other than staphylococci.

Table 1 The number of selected lactic acid bacteria (LAB) and coagulase-negative staphylococci (CNS) from Kung-Som

Medium	Acid-producing bacteria	Selected LAB/bacteria	Shape		
			Rod	Cocci	CNS
MRS	120	96	76	20	-
M17	120	71	12	59	-
MSA	120	116	-	-	116

* Note MRS = de Man Rogosa and Sharpe ; M17 = M17 ; MSA = Mannitol Salt Agar

Primary screening of glutaminase-producing strain

Agar rapid plate method was carried out for screening glutaminase-producing LAB and CNS bacteria. All 167 strains of LAB and CNS bacterial cultures were grown on the modified MRS medium and glutamine medium supplemented with phenol red as indicator which it gives yellow color to media (in acidic and neutral pH), and it exhibits the pink color when the pH changes to alkaline condition. The pink zone around bacterial colony (Fig. 1) indicates the ammonia accumulation strains (Sathish *et al*., 2010). It was found that only 80-LAB isolates (48%) and 36 isolates (31%) exhibited pink color around colony which indicated ammonia accumulate strains (Fig. 1).

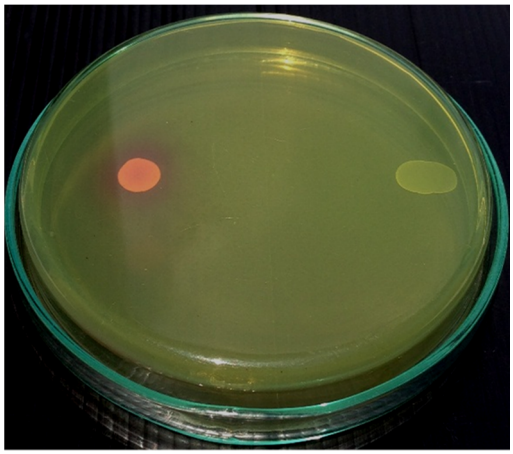

Figure 1: Glutaminase-producing bacteria on glutamine medium or modified MRS medium after incubation at 37 ºC for 24 h.

In addition, all 116 strains were tested for glutaminase production by TLC. The TLC were used for the separation and identification of glutamic acid produced by glutaminase-producing strains and were estimated by redness spot development after spraying with ninhydrin reagent. The Glutamic acid is a compound produced (Fig.2 lane 1), after hydrolysis of glutamine medium (Fig.2 lane 2) by glutaminase (Siddalingeshwara *et al*., 2010). It was found that only 38-LAB strains and 9-CNS bacteria strains were exhibited the spot in the same R_f value of standard glutamic acid (Fig. 2).

Determination of L-glutaminase activity

Determination of glutaminase activity of LAB (38 strains) and CNS bacteria (9 strains) using Nessler's reagent was carried out. Results showed that LAB strains J01SM17 and J01SM28 showed the glutaminase activity at 0.145 U/ml and 0.125 U/ml, respectively (Table 2). In addition, CNS bacteria strains J03SM3 and J03SM5 exhibited the glutaminase activity at 0.328 U/ml and 0.376 U/ml, respectively. Accordingly, they were selected as the glutaminase-producing strains for further study.

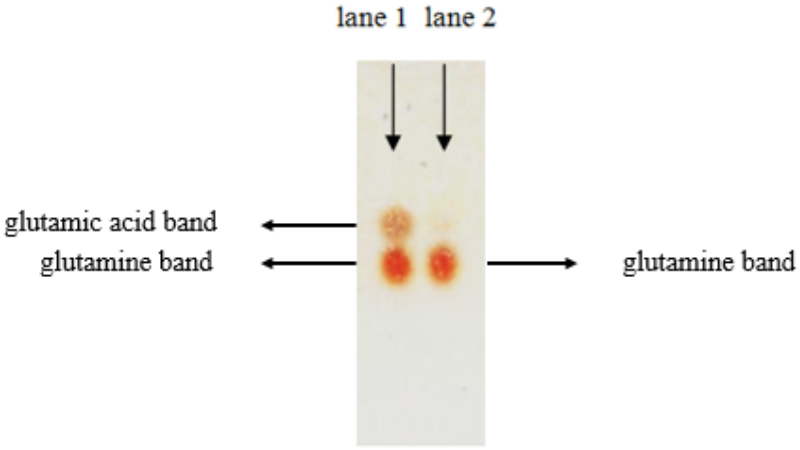

Figure 2: Detection of glutamic acid by thin layer chromatography (TLC). Supernatant (2 µl) was subjected to TLC (lane 1), lane 2 is glutamine medium.

Identification of glutaminase-producing strain

All 4 strains (J01SM17, J01SM28, J03SM3 and J03SM5) were identified by 16S rDNA gene sequencing. It was found that LAB strains J01SM17 and J01SM28 were identified as *Lactobacillus rhamnosus* and CNS bacteria strains J03SM3 and J03SM5 were identified as *Staphylococcus piscifermentans* and *Staphylococcus saprophyticus* (Table 2). A dendogram constructed using the neighbour-joining method for the 16S rDNA sequence of the isolates and their closest relatives is shown in Fig 3.

Table 2 Identification and glutaminase activity of selected LAB and CNS

Strain	Closely related strain	Identification (%)	Glutaminase activity (U/ml)	
			pH 7.0	pH 5.0
J01SM17	*Lactobacillus rhamnosus*	100	0.145 ± 0.0062	0.082 ± 0.0075
J01SM28	*Lactobacillus rhamnosus*	99	0.125 ± 0.0070	ND
J03SM3	*Staphylococcus piscifermentans*	99	0.328 ± 0.0035	0.186 ± 0.0700
J03SM5	*Staphylococcus saprophyticus*	100	0.376 ± 0.0096	ND

ND = not detected; Values are the average of three measurement.

The pH dependence of glutaminase activity of all selected strains was determined at pH 5.0 and 7.0 using Nessler's reagent. The optimum pH for the glutaminase production were shown in Table 2. These results were similar to the optimum pH of glutaminase production from *L. rhamnosus* and *L. reuteri* KCTC3594 (Weingand-Ziadé *et al*., 2003; Jeon *et al*., 2010). Although, *Staphylococcus saprophyticus* exhibited the highest glutaminase activity (0.376

U/ml), it was not chosen for starting culture in Kung-Som production, because of it contained antibiotic resistant gene and had reported as a pathogenic bacteria in human (Ferreira *et al*., 2012).

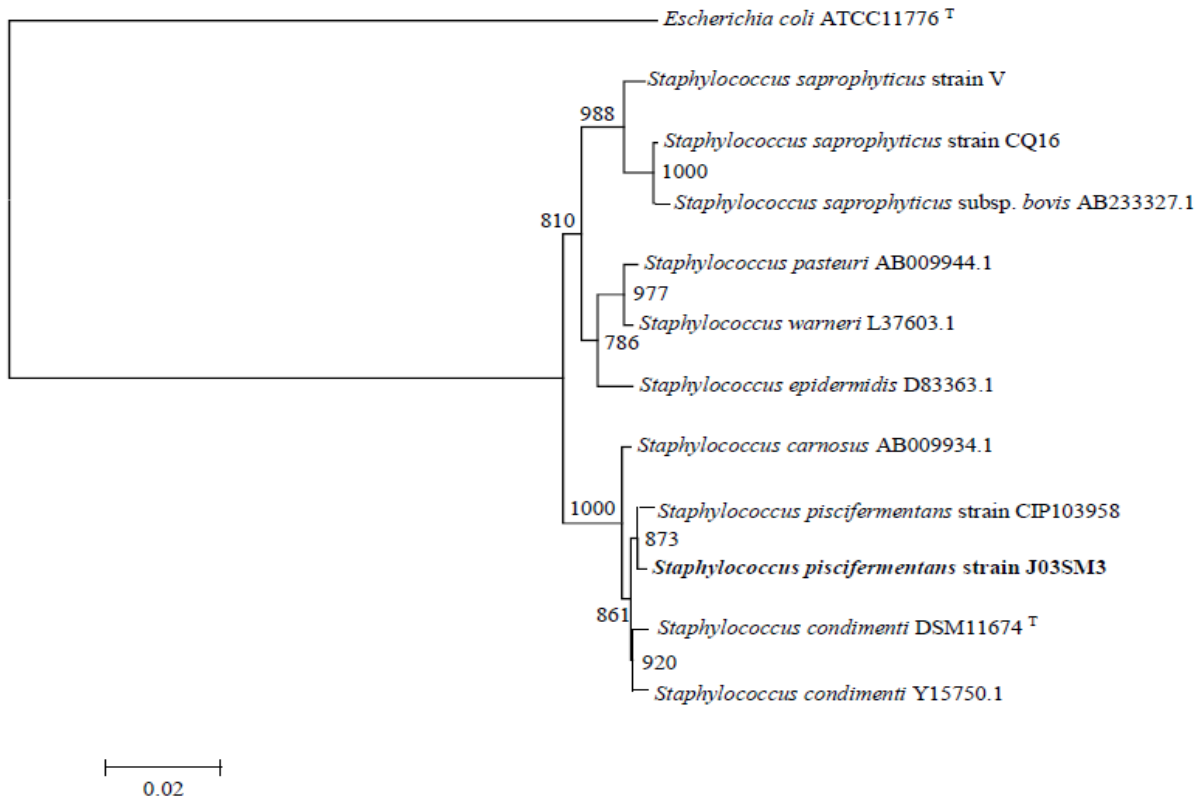

Figure 3: Phylogenetic tree for the glutaminase-producing CNS bacteria strain J03SM3, isolated from Kung-Som on the basis of 16S rDNA gene sequences.

Conclusion

Glutaminase-producing LAB and CNS were successfully isolated from Kung-Som. We can use these strains as starting cultures to increase the glutamic acid content for enhancing flavors in Kung-Som production.

Acknowledgements

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