

Determination Amylolytic Characteristic of Predominant Lactic Acid Bacteria Isolated during Growol Fermentation, in a Different Starch Medium Composition

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ABSTRACT

In order to achieve efficient lactic acid production from starch, fermentation of avarious composition starch medium by lactic acid bacteria was examined in this study. Many strains of *Lactobacillus plantarum* isolated from growol fermentation, *Lactobacillus plantarum* subsp. *plantarum* NBRC 15891 and *Lactobacillus amylophyllus* NBRC 15881 were used as starter cultures in starch basis medium, i.e, basal, basal-starch, enriched basal-starch with polypeptone and yeast extract. *Lactobacillus plantarum* UA3, AA2, AA11 showed the highest cells growth compare to both reference strains, but *Lactobacillus amylophyllus* NBRC 15881 showed a greater ability to degrade starch indicated by decreasing of pH and starch content of the fermented substrate. Enriched medium with peptone and yeast extract could generate the growth and starch degradation capabilities for all types of lactic acid bacteria were used.

Keywords: Growol; Lactic Acid Bacteria; Amylolytic Characteristics.

INTRODUCTION

Amylolytic activity of fermenting organism is a major characteristic for fermentation of starch to lactic acid. Lactic acid bacteria which have ability to utilize starch is known as amylolytic lactic acid bacteria (ALAB). Due to the ability to partially hydrolyze raw starch (Reddy et al., 2000), ALAB can ferment different types of

amylaceous raw material, such as corn (Nakamura, 1981), potato (Chatterjee et al., 1997), or cassava (Giraud et al., 1994) and different starchy substrates (Vishnu et al., 2000, 2002; Naveena et al., 2003). Some strains of *Lactobacillus spp.* produce extracellular amylase and ferment starch directly to lactic acid. The activity of lactic acid bacteria in fermented starchy materials contributed to the production of lactic acid, specific enzymes, and aromatic compounds (Camargo et al., 1988; Demiate et al., 1999; Marcon et al., 2006).

Growol is an Indonesian traditional and complementary food prepared from cassava and is made by soaking fresh peeled cassava roots for 3-5 days, followed by filtering to get starch and pressing to decrease moisture content, and then is steamed to make "ready to eat" growol. During fermentation, many kinds of microorganisms changed daily, i.e., a group of *Streptococcus*; *Coryneform*; yeast and a group of *Enterobacteriaceae*; *Bacillus sp* and *Acinetobactersp*; *Lactobacillus sp* and *Moraxella sp* (Rascana, 1986). Although there are many kinds of microorganism involved, *Lactobacillus plantarum* is the predominant LAB which grew from the beginning to the end of fermentation (Rahayu, 1992). The fact that starch is the main carbohydrate in cassava led to the idea that ALAB could grow during growol fermentation. Therefore, an investigation of amylolytic characteristic of *Lactobacillus plantarum* isolated from different day of

fermentation is necessary in order to obtain indigenous isolates for using as a starter culture in the lactic acid production from cassava starch or another uses.

For growth, lactic acid bacteria require nutrients, some vitamins, minerals, amino acids and certain peptides as a nitrogen source and has the ability to use carbohydrate as an energy source. Medium components must fit the requirements for cell biomass and metabolite production and meet the adequacy requirement of energy for biosynthesis and cell maintenance (Calderon et al, 2001). Based on the facts that cassava starch as fermentation substrate does not contain enough nitrogen needed for growth of lactic acid bacteria, this study investigates the effect different enriched-starch based medium for strains growth.

MATERIAL AND METHODS

Bacterial strains and culture condition.

The microorganism used were *Lactobacillus plantarum* subsp. *plantarum* AA11, AA2 and UA3 isolated from soaking cassava during *growol* fermentation (Putri et al., 2010) and *Lactobacillus amylophyllus* NBRC 15881 (NITE Biological Resources Center, Chiba, Japan). These lactic acid bacteria have been used throughout this study.

The colony of isolates were picked up from MRS-agar plate and their morphology were observed by microscope at enlargement 1000x. The colony colors of isolates were observed at the pellet of 24 h isolates. Gas producing ability of isolates were observed for 72 h incubation in Glucose Yeast Pepton (GYP) broth media; glucose 2 g l⁻¹, yeast extract 1 g l⁻¹, peptone 1 g l⁻¹, Na-acetate. 3H₂O 0.5 g l⁻¹, salts solution (MnSO₄.4H₂O 2 mg/ml, MgSO₄.7H₂O 40 mg/ml, FeSO₄.7H₂O 2

mg/ml and NaCl 2 mg/ml) 0.5 ml l⁻¹, Tween 80 solution 1.0 ml l⁻¹ in pH 6.8.

The ability of isolates to hydrolyzed starch were observed using modified MRS Agar medium (DeMan et al., 1960). MRS starch composition has been used (in g l⁻¹): soluble starch: 20; bactopectone: 10; beef extract: 10; yeast extract (Difco): 5; diammonium hydrogen citrate: 2. anhydrous sodium acetate: 5; K₂HPO₄: 2; MgSO₄.7H₂O: 0.20; MnSO₄.4H₂O: 0.2 and Agar: 15. After incubation for 72 hours, agar plates were poured with Gram's iodine solution and observed the clear zone surrounding of isolates colony. The ability of isolates to grow in different composition medium also measure by using (1) Basal media (as used for API system, Bio-Mé'rieux, France) in g l⁻¹: polypeptone, 10; yeast extract, 5; K₂HPO₄, 2; sodium acetate, 5; MgSO₄.7H₂O: 0.20; MnSO₄.4H₂O: 0.05, tween 80 1 ml, (2) Basal-starch media was contain basal media with soluble starch (in g l⁻¹): soluble starch, 10; polypeptone, 10; yeast extract, 5; K₂HPO₄, 2; sodium acetate, 5; MgSO₄.7H₂O: 0.20; MnSO₄.4H₂O: 0.05, tween 80 1 ml, (3) Basal-starch media omitting yeast extract. (4) Basal-starch media omitting peptone.

Fermentations were performed at 37°C with initial pH was adjusted to 6.5. The medium was inoculated (10% v/v) with 24 h precultures grown on MRS. During fermentation, a 4 ml sample were collected once in 0, 4, 24 and 48 h under aseptic conditions.

Growth measurement and pH.

Optical density at 600 nm (O.D.₆₀₀) was measured using a Spectronic 401 spectrophotometer (Guyot and Morlon-Guyot, 2001). For estimation of pH, fermented broth was taken in falcon tubes and centrifuged at 7,500 rpm for 10

minutes to pellet out the bacterial growth. pH was determined by pH meter.

Starch hydrolysis test.

1. Qualitative analysis.

Different isolates were streaked on to individual MRS agar plates and were incubated at 37°C. Then from each plate isolated colonies were picked up and streaked in straight lines in starch agar plates with starch as the only carbon source (Diaz-Ruiz et al., 2003). After incubation at 37°C for 24-48 hours, individual plates were flooded with Gram's iodine (Gram's iodine- 0.15% iodine crystals added to 1.5% potassium iodide solution. Stored at room temperature) to produce a deep blue colored starch-iodine complex. If a strain is amylolytic then it starts hydrolyzing the starch present in the plate nearby its growth and in the zone of degradation no blue color forms, which is the basis of the detection and screening of an amylolytic strain. The zone of decolorization becomes visible within few seconds of addition of I₂-KI solution and removing excess of the solution. Along with these isolate the standard strain was also subjected to this test.

2. Quantitative analysis.

Only those isolates (a total of 4) that give a positive result in qualitative starch hydrolysis test were subjected to this quantitative test (the method of Nakamura, 1981 as described by Calderon et al., 2003). For this, individual isolates were grown on the respective media broth for 24 hours and then an aliquot of 2 ml was withdrawn at regular intervals of 4 hours and starch degradation profile was established. For this 1ml of broth was centrifuged to pellet out bacterial growth. Then the supernatant in

each case was diluted 100 times with distilled water. To 10 ml of this 1 ml of Gram's iodine was added in a test tube. The mixture was vortexed and the absorbance of the resultant blue colored complex was measured at 585 nm with a spectrometer. The concentration of the residual starch in each case was worked out from a standard curve.

RESULTS AND DISCUSSION

General characteristics of LAB strains.

Strains isolated during growol fermentation showed differences on colony color, physiological characteristics (pH and temperature of growth) and their ability to grow in MRS media. *L. plantarum* AA2, *L. plantarum* AA11, *L. plantarum* UA3 and *L. amylophylus* NBRC 15881 were homofermentative where lactic acid is the major product from glucose and they also displayed a typical rod-shape cell structure. The taxonomy of lactic acid bacteria has been based on the Gram reaction and the production of lactic acid from various fermentable carbohydrates. Lactobacilli vary in morphology from long, slender rods to short coccobacilli, which frequently form chains. (Axelsson, 2004). Strains of *Lactobacillus plantarum* have been isolated from African cassava-based fermented products (Nwankwo et al., 1989). Recently, Sanni et al. (2002) described amylolytic strains of *L. plantarum* and *L. fermentum* strains in various Nigerian traditional amylaceous fermented foods. The search for ALAB in fermented amylaceous foods has been justified by the high starch content of the raw material.

Table 1. General Characteristics and Growth Ability of Strains.

Characteristics	<i>L. amylophylus</i> NBRC 15881	<i>L. plantarum</i> AA2	<i>L. plantarum</i> AA11	<i>L. plantarum</i> UA3
I. Morphological and culture characteristics.				
• Colony's color	Transparent	Opaque yellowish	Opaque yellowish	Opaque white yellowish
• Shape	Short rod	Short rod	short rod	Short rod
• Development on solid medium	Smooth round colonies	Smooth round colonies	Smooth round colonies	Smooth round colonies
• Development on liquid medium	Uniform turbidity, sediment formation	Uniform turbidity, sediment formation	Uniform turbidity, sediment formation	Uniform turbidity, sediment formation
II. Physiological characteristics.				
pH				
3	+	+	+	+
4	++	++	++	++
5	++	++	++	++
temperature(°C)				
10	+	+	+	+
37	++	++	++	++
45	-	-	+	+
III. Biochemical Characteristics.				
Fermenting type	homofermentative	homofermentative	homofermentative	heterofermentative
Catalase production	negative	negative	Negative	Negative
IV. Growth on MRS-starch media				
Growth ability	+	+	++	++
Starch hydrolysis	w	w	w	w

*) after 48 hours of incubation, + have positive ability, ++ have greater positive ability w: weak ability.

All of isolates grew well in MRS-starch agar media although have weak amylolytic characteristic, included *Lactobacillus amylophylus* NBRC 15881 as amylolytic reference strain (Table 1). These strains capability could be provided because MRS media contained many kinds of nutrition which can be used by isolates. These suggested that nutrition in media influence the strains growth. They could not show a clear zone surrounding their colony that concluded that all of isolated strains had weak ability to degrade starch. These isolates were isolated only five days of fermentation and during that times there were other kind

of microorganism which could supply glucose as a carbon source (Rascana, 1986). *Lactobacillus amylophylus* NBRC 15881 as an amylolytic type isolates was a 'slow growing' lactic acid bacteria and needed more time of incubation to show its ability to degrade starch.

Amylolytic Characterization.

The comparison between the growth of isolates in different composition media showed that the isolates could grow better if the medium of fermentation contained starch that used as the only carbon source (Figure 1) and the pH of medium decreased greater in basal-starch medium (4.3) than in

basal medium (6.1). An addition of nitrogen sources such as yeast extract with a concentration of 1%, have shown to promote cell growth (Figure 1) and starch degrading ability (Figure 2). Basal starch media resulted in increasing *L. amylophylus* NBRC 15881 cell growth more than threetimes higher than in basal media (0.21 becoming 0.82 absorbance at 600 nm), and also enhanced one to two times cell growth of another three strains. LAB is a type of bacteria that require complex nutrients for growth such as amino acids, peptides, nucleotide bases, vitamins, minerals, fatty acids and carbohydrates. In addition, LAB produce small quantities of organic compounds that provide aroma and flavor to the products of fermentation when there are enough nutrients in growth media (Axelsson, 2004; Ercolini et al., 2001; Holzapfel et al., 2001; Jay, 2000).

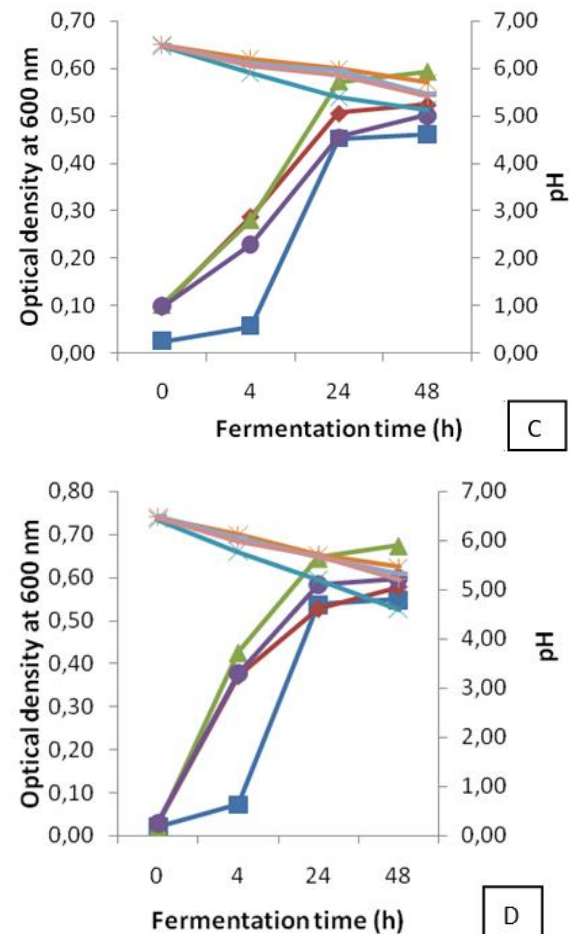
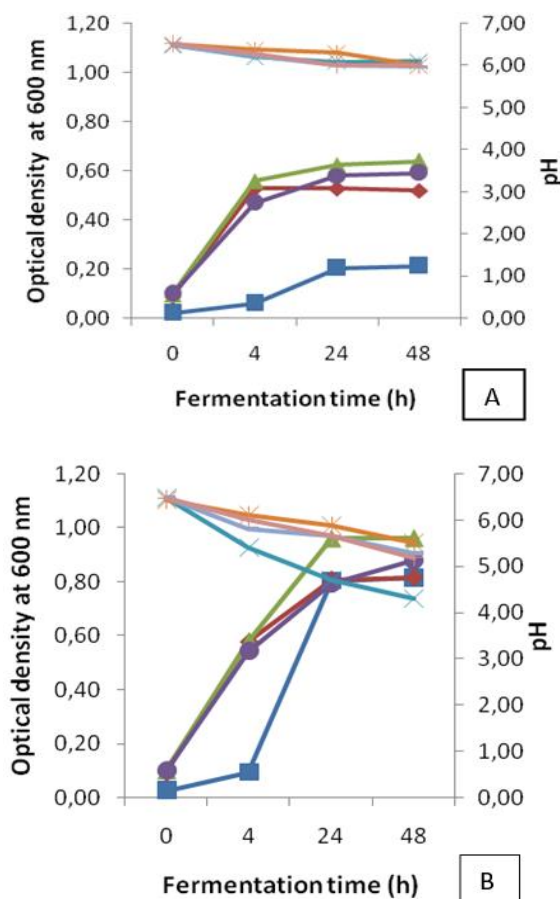


Figure 1. Growth ability of strains at 37°C with pH control in: A. basal media, B. basal starch media, C. basal starch media without yeast extract, D. basal starch media without peptone. Symbols: (■) growth of *L. amylophylus* NBRC 15881, (◆) growth of *L. plantarum* AA2, (▲) growth of *L. plantarum* AA11, (●) growth of *L. plantarum* UA3, (x) pH of *L. amylophylus* NBRC 15881, (+) pH of *L. plantarum* AA2, (-) pH of *L. plantarum* AA11, (+) pH of *L. plantarum* UA3.

Basal-starch-yeast extract (omitting peptone) gave a better result on bacterial growth, resulted lower pH than basal-starch omitting yeast extract. Even, this media showed similar results to the basal media which contained yeast extract and peptone. Xiaodong et al. (1997) showed different results, whereas the addition of peptone resulted LAB cell growth better than the addition of yeast extract on the fermentation of cassava starch by

Lactobacillus amylovorus, characterized by increased glucose consumption and high lactic acid concentration at the end of fermentation. Conversely, Djeghri-Hocine et al. (2006) stated that starch media contain horse bean extract supplemented with yeast extract was able to enhance the growth of *L. plantarum* isolated from plants, compared to growth in MRS medium containing peptone and yeast extract. While Guyot and Morlon-Guyot

(2001) showed that meat extract which added in soluble starch medium (replacing minerals) produce better growth than the addition of yeast extract or pancreatic peptone, with an increase of 15%, 62% and 26%. The results of different studies on medium supplementation effect showed that the type of nutrients needed by LAB is specific to each type and the medium from where BAL is isolated.

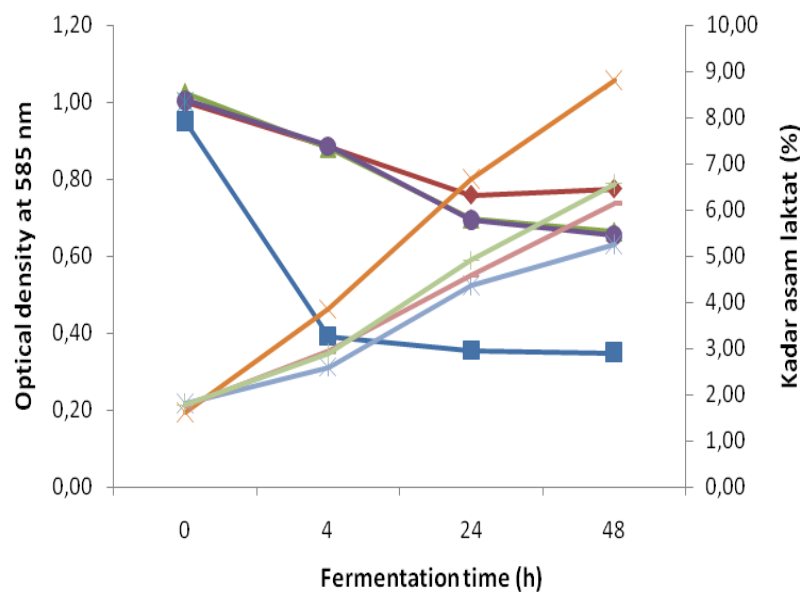


Figure 2. Starch degrading ability of strains at 37°C with pH control (6.5) in basal starch without peptone media. Symbols: (■) OD 585 of *L. amylophyllus* NBRC 15881, (◆) OD 585 of growth of *L. plantarum* AA2, (▲) OD 585 of *L. plantarum* AA11, (●) OD 585 of *L. plantarum* UA3. (x) lactic acid content of *L. amylophyllus* NBRC 15881, (*) lactic acid content of *L. plantarum* AA2, (-) lactic acid content of *L. plantarum* AA11, (+) lactic acid content *L. plantarum* UA3.

Lactobacillus amylovorus was isolated from BAL-fortified corn waste and containing peptides, therefore this LAB requires for its growth stimulators such as those found in maize (Xiaodong et al., 1997). Guyot and Morlon-Guyot (2001), also stated that a more complete nutrition in the form of nitrogen source and 'growth factor' in the media, these would give positive effect on LAB cell growth and its ability in the production of lactic acid. Cassava starch is a nutrient-poor substrate, so the addition of nitrogen in the form of a combination of amino acids and B vitamins as found in yeast extracts, will be able to increase its growth better. Busairi (2010) add 5g/ (0.5%) yeast extract in medium pineapple extract and suggests that yeast extract can enhance the growth of *Lactobacillus delbrueckii* subsp. *delbrueckii* ATCC 9649 much greater than other nitrogen sources. The amount or concentration of nutrients were added on a medium is highly dependent on the main substrate and the type of media where the LAB was isolated.

Lactic acid bacteria being fastidious organisms require amino acids and vitamins for their growth, therefore higher level of nitrogenous compound was required (Altaf et al., 2007). Effect of nitrogen concentration required for lactic acid production by *L. casei* has been reported by Guyot et al. (2003), they found that the presence of peptides in yeast extract enhanced the growth of *Lactobacillus*. Yeast extract rich in B vitamins is known to enhance lactic acid production rates by lactic acid bacteria explained the contribution of B vitamins, purines and pyrimidine bases in medium for growth of *Lactobacillus*.

The growth of *L. plantarum* subsp. *plantarum* AA2, AA11, and UA3 were faster than *Lactobacillus amylophyllus* NBRC 15881, but they could not decrease pH as low as that be done by *Lactobacillus amylophyllus*. This amylolytic reference strain could efficiently used starch and produced more lactic acid as the pH was decreased greater. These results could be proved that all of isolates from cassava fermentation had ability to use starch for growth and produced lactic acid although their ability was quite the same with *L. plantarum* type isolates. Calderon et al. (2001) were observed the production of amilase enzyme from *Lactobacillus fermentum* strain Gi E1, his results showed that the growth and amylase production of this lactic acid bacteria more greater in maltose-starch compare with only starch as a substrate. These could be explain that starch conversion was limited by accumulation of limit dextrin which can not further fermented by lactic acid bacteria, thus the growth and amylases synthesis limited also. Therefore, it still remains to be determined if such isolates would be able to simultaneously express these metabolic activities in complex food matrixes and produces lactic acid.

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