

Growth and Survival Rates of *Lactobacillus plantarum* in Thai Cereal Cultivars

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Received: 19 June 2014; Accepted: 28 July 2014; Published online: 6 August 2014

DOI: 10.14416/j.ijast.2014.07.005

Abstract

In this study, the viability of probiotic Lactobacillus plantarum TISTR 2075 in different kind of cereal extracts was determined during fermentation and storage. During storage at 4°C for 144 h, fermented Plai Ngahm Prachin Buri rice extract exhibited the highest survival rate of 94.91% which was significant difference ($P < 0.05$) from other fermented cereal extracts. Whereas, the highest survival rate of the strain stored at 37°C for 60 h was detected in fermented black glutinous rice extract (83.40% survival rate). Furthermore, the stability of the strain could be considered in a term of the specific rate of degradation (k). The lowest k value of 0.0034 h^{-1} and 0.0181 h^{-1} was also observed in fermented Plai Ngahm Prachin Buri rice extract storage at 4°C and fermented black glutinous rice extract storage at 37°C, respectively. Total reducing sugar and free amino nitrogen evolution of all fermented cereal extracts decreased over the course of storage period.

Keywords: Probiotic, Fermentation, Storage stability, Cereal extracts

1 Introduction

Recently, consumers across the world are becoming more interested in foods with health promoting features as they can gain more awareness of the links between food and health [1]. Functional foods are foods or food ingredients that exert a beneficial effect on host health and/or reduce the risk of chronic disease beyond basic nutritional function [2-5]. This term cover a broad range of products such as probiotic yoghurt, cholesterol-lowering spreads and oligosaccharide-added foods [2]. Probiotics are defined as live organisms that when administered in adequate amount ($> 6\text{-}7 \log \text{ CFU/g}$) confer health benefits to the host [6]. The application of

probiotic cultures in non-dairy products represents a great challenge and needs to be researched at the industrial level for commercial production of the healthy products [7,8]. Normally, probiotics have been added to fermented dairy products. Nowadays, an increased demand for non-dairy probiotic products comes from vegetarianism [9]. The allergy to dairy products affects negatively some persons. Moreover, lactose intolerance and the cholesterol content are two major drawbacks related to the fermented dairy products [10]. These have led to development of probiotic products from various food matrices including fruits, vegetables and cereals [7,9]. In particular, cereals have been researched as suitable substrates to stimulate the growth of

probiotic microorganisms [11]. Several studies have been using cereals or cereal extracts as potential growth medium for probiotic [4,8,12-15]. Rathore et al. [13] revealed that malt proved to be a great substrate for the growth of *Lactobacillus plantarum* NCIMB 8826 and *Lactobacillus acidophilus* NCIMB 8821. These results were in accordance with Salmerón et al. [16] that *L. acidophilus* NCIMB 8821 exhibited the highest cell population of approximately 8.5 log CFU/mL in malt substrate. While, *L. plantarum* NCIMB 8826 achieved the highest cell number of 8.2 log CFU/mL and 7.9 log CFU/mL in oat and barley media, respectively. Furthermore, rice bran was used as culture medium for the growth of *L. casei* and *L. plantarum* B2 with the viable cell number of 9.03 log CFU/mL and 9.09 log CFU/mL, respectively [17]. Also, germinated Pearl millet could be used as the growth medium of *L. acidophilus* (NCDC-16) with the viable cell count of 8.64 log CFU/mL [18].

The objective of this study was to see the applicability of using four varieties of Thai cereal cultivars as a suitable substrate for the growth of probiotic lactic acid bacteria. The stability of the strain in fermented cereal extracts during storage was investigated in a term of the specific rate of degradation. Furthermore, free amino nitrogen (FAN), total reducing sugar (TRS), lactic acid content and pH of fermented cereal substrates were also determined.

2 Materials and Methods

2.1 Microorganisms

The probiotic strain, *Lactobacillus plantarum* TISTR 2075 isolated from fermented vegetables was obtained from Microbiological Resource Center, Thailand Institute of Scientific and Technological Research (TISTR), Thailand. The strain was preserved in de Man-Rogosa-Sharpe (MRS) broth (Difco, Detroit, MI, USA) with 20% (v/v) glycerol content at -20°C. For routine analysis, the strain was subcultured twice in MRS broth and was incubated at 37°C for 24 h under microaerobic-static conditions to maintain freshness and used as inoculum.

2.2 Preparation of cereal extracts fermentation and storage

Fermented cereal extracts were prepared according to the procedures described by Wang et al. [19]. Cereals (Plai Ngahm Prachin Buri rice, black glutinous rice, White kidney bean and Red kidney bean) were washed and soaked in distilled water. The soaked cereals were mixed with distilled water (cereal:water = 1:10 w/v). After decanting the soaking water, the soaked cereals were mixed with distilled water and then comminuted in a blender for 3 min [20]. The resultant slurry was filtered through double-layered cheesecloth 2 times to yield cereal extracts. Each of cereal extracts was dispensed into containers and sterilized by heating at 121°C for 15 min. Sterilized cereal extracts were inoculated with overnight culture of 1% (v/v) of *L. plantarum* TISTR 2075. The fermentations were performed under no pH control in Duran screwcapped glass bottles at 37°C for 24 h. Viable cell counts were determined by the standard plate count method with MRS medium supplemented with 0.5% CaCO₃ at 37°C. pH was measured with a pH meter.

During storage period, fermented cereal extracts containing probiotic *L. plantarum* TISTR 2075 were stored at 4°C for 144 h and 37°C for 60 h. The specific rate of degradation (k , h⁻¹) was calculated as first-order reaction from $k = \frac{1}{t} \times (\log_{N_0} - \log_{N_t})$, where N refers to the bacterial count at a particular storage period (CFU/mL), N₀ represents the bacterial count at the beginning of the storage (CFU/mL) and t is the storage time. The viable cell counts, pH, lactic acid content, reducing sugar and free amino nitrogen of fermented cereal extracts were determined.

2.3 Analytical procedure

2.3.1 Viable cell counts

Viable cell counts were determined by the standard plate count method on MRS agar plate. The plates were incubated at 37°C for 24 h. The viable cell counts were expressed as log₁₀ value/mL. The percentage of cell survival was defined as follows: survival rate (%) = $\left(\frac{N}{N_0}\right) \times 100$, where N represents the number of viable cells count (log CFU/mL) at a particular period and N₀ denotes the initial viable cell count (log CFU/mL) at the beginning [21].

2.3.2 Lactic acid content

The supernatant of culture (2 mL) mixed with distilled water (18 mL) was titrated with 0.1 M NaOH. Phenolphthalein (1 mL) was used as an indicator. Each milliliter of 1 N NaOH is equivalent to 90.08 mg of lactic acid. The titratable acid was then calculated according to AOAC method [22].

2.3.3 Reducing sugar

Reducing sugars were determined by the 3, 5-dinitrosalicylic acid (DNS) colorimetric method [23], with glucose as the standard.

2.3.4 Free amino nitrogen

The concentration of free amino nitrogen (FAN) was estimated by the ninhydrin colorimetric method (European Brewery Convention) [24] using glycine solution as control.

2.3.5 Statistical analysis

Each result was expressed as the mean \pm S.D. of three determinations. The data were assessed using analysis of variance (ANOVA) with a level of significance at $P < 0.05$. Significant divergences among mean values were determined with Duncan's multiple range tests. All statistical analyses were performed using SPSS Software, version 12 (SPSS, now a part of IBM Corp.; White Plains, NY, USA).

3 Results and Discussion

3.1 Fermentation of cereal extracts by *L. plantarum* TISTR 2075

In the present study, Plai Ngahm Prachin Buri rice extract, black glutinous rice extract, White kidney bean extract and Red kidney bean extract were used as culture media for the growth of *L. plantarum* TISTR 2075. As shown in Figure 1, *L. plantarum* TISTR 2075 grew well in White kidney bean extract providing the viable cell number of 8.66 log CFU/mL followed by Red kidney bean extract (8.56 log CFU/mL), Plai Ngahm Prachin Buri rice extract (7.77 log CFU/mL) and black glutinous rice extract (7.60 log CFU/mL),

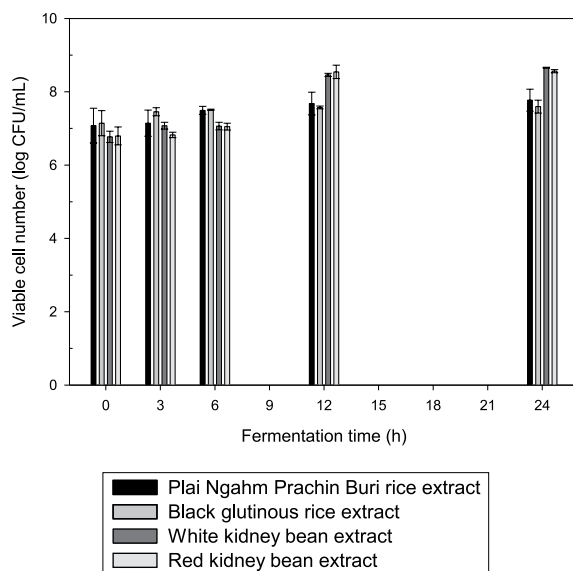


Figure 1: Viable cell number of *L. plantarum* TISTR 2075 in fermented cereal extracts during fermentation at 37°C for 24 h.

respectively. The highest increase in viable cell number of 1.89 log CFU/mL after 24 h fermentation at 37°C was observed in fermented White kidney bean extract followed by 1.77 log CFU/mL in fermented Red kidney bean extract. The slightly increase in viable cell count of 0.69 and 0.45 log CFU/mL were exhibited in fermented Plai Ngahm Prachin Buri rice extract and fermented black glutinous rice extract, respectively. Similar to all living organisms, microorganism requires carbon source from carbohydrates, nitrogen source from protein, trace elements and vitamins to build up its own biomass [25]. From the chemical composition of cereal cultivars presented in Table 1, carbohydrate content of all varieties was high considered as good source of carbohydrate. Among all varieties, White kidney bean and Red kidney bean exhibited higher protein values than that of rice cultivars. It could be implied that rich source of carbohydrate and protein in White kidney bean and Red kidney bean may resulted in high viable cell number of probiotic *L. plantarum* TISTR 2075. This was in agreement with Kedia et al. [25], that the available carbohydrates and amino acids in protein may also contributed to growth increase, higher cell viability and greater production of lactic acid.

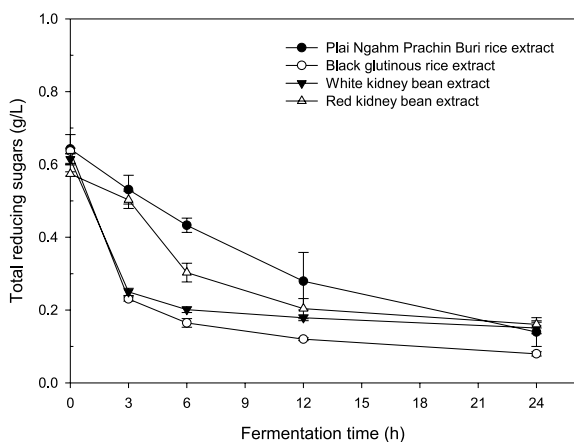


Figure 2: Total reducing sugar concentration during the fermentation of different cereal extracts at 37°C for 24 h.

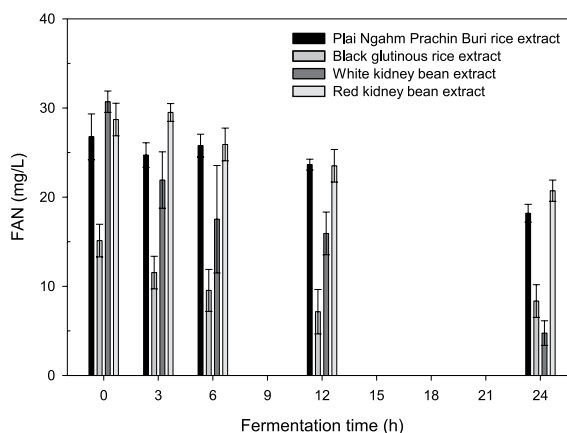


Figure 3: Free amino nitrogen (FAN) evolution during the fermentation of different cereal extracts at 37°C for 24 h.

Table 1: Chemical composition of cereal cultivars (g/100 g DM basis)

Variety	Moisture	Protein	Fat	Crude fiber	Carbohydrate	References
Rice ^a	11.14 ± 0.06	8.87 ± 0.06	2.92 ± 0.03	1.12 ± 0.04	74.23 ± 0.02	[26]
Black glutinous rice	12.59 ± 0.16	8.17 ± 0.41	3.72 ± 0.06	4.01 ± 0.58	74.09 ± 0.48	[27]
White kidney bean	-	25.63 ± 0.35	1.30 ± 0.02	-	69.62 ± 0.38	[28]
Red kidney bean	12.39 ± 0.60	21.83 ± 2.80	1.30 ± 0.33	-	60.65 ± 2.21	[29]

^aJasmine rice Khao Dok Mali 105

As shown in Figure 2 and 3, it was observed that the total reducing sugars (TRS) and free amino nitrogen (FAN) profiles were considerably different in the cereal extracts. The values of TRS and FAN in fermented cereal extracts tended to decrease through 24 h fermentation. The initial TRS concentration of all cereal extracts ranged from 0.57 to 0.64 g/L. After fermentation at 37°C for 24 h, fermented black glutinous rice extract exhibited a great reduction in TRS of approximately 0.55 g/L followed by fermented Plai Ngahm Prachin Buri rice extract (0.50 g/L reduction), White kidney bean extract (0.46 g/L reduction) and Red kidney bean extract (0.41 g/L reduction), respectively. The decrease in TRS concentration could be attributed to the consumption of fermentable sugars as carbon source during the growth of microorganisms [30]. Rathore et al. [13] suggested that *L. plantarum* exhibits specific preference towards glucose.

In addition, the initial FAN concentration of all cereal extracts were 15.13-30.70 mg/L. Among all fermented cereal extracts tested, the reduction of

FAN in fermented White kidney bean extract after 24 h fermentation was found to be 84.53% which was higher than that of fermented black glutinous rice extract (44.85% reduction), fermented Plai Ngahm Prachin Buri rice extract (32.04% reduction) and fermented Red kidney bean extract (27.82% reduction), respectively. This result was in close agreement with the finding of Kedia et al. [25] that FAN of fermented malt suspension decreased linearly to minimum of 21.6 mg/L during 26 h fermentation, even throughout the stationary phase to provide maintenance energy for the cells. Lactic acid bacteria strain could use the amino acids and peptides available to support growth or they degrade the proteins of the cereal substrates through their proteolytic systems increasing the amounts of amino acids and peptides in the culture media [16].

The pH value and lactic acid content evolve in a different manner depending on type of cereal extracts. The lactic acid production of 0.05-0.11% resulted in a decrease of broth pH which dropped from 6.41 to 3.93 throughout 24 h fermentation (Figure 4). A drop in pH

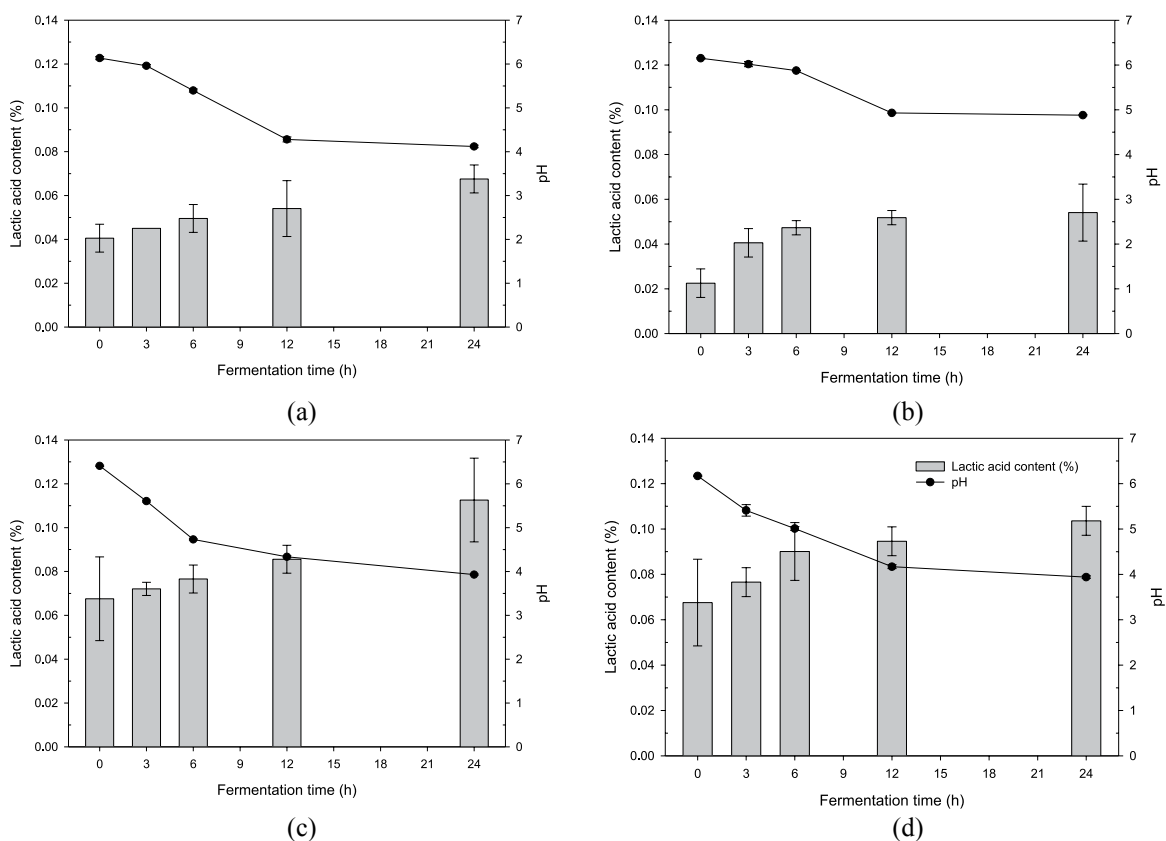


Figure 4: Change in pH and lactic acid content in fermented Plai Ngahm Prachin Buri rice extract (a), fermented black glutinous rice extract (b), fermented White kidney bean extract (c), and fermented Red kidney bean extract (d) at 37°C for 24 h.

with corresponding increase in titratable acidity has been reported in lactic acid fermentation of a number of foods including barley [30], oat [31] and cereal-legume blend [15]. Arora et al. [30] also suggested that the homo-fermentative *L. acidophilus* converts glucose to lactic acid, which is responsible for the decline in pH of the product. Furthermore, production of lactic acid during fermentation by microorganisms can be affected by medium compositions such as carbohydrate source, sugar concentration and growth factor [32].

3.2 Storage stability of *L. plantarum* TISTR 2075 fermented in cereal extracts

To exert beneficial effects in the host, it is important that probiotic lactic acid bacteria could be alive and abundant in the product at the time of consumption [33]. Cell viability and survival rate of *L. plantarum* TISTR 2075 in

different fermented cereal extracts during storage at 4°C for 144 h and 37°C for 60 h were shown in Figure 5-6. Evidently, the strain in fermented cereal extracts kept at 4°C could be able to remain its viability at high number (> 7 log CFU/mL) until the end of storage. This was well above the recommended therapeutic minimum of 6 log CFU/mL at the time of consumption [34,35]. The survival rate of 86.73-94.91% was observed at 4°C storage which was higher than that of 37°C storage (70.49-83.40% survival rate). At 4°C, the strain fermented in Plai Ngahm Prachin Buri rice extract exhibited the highest survival rate of 94.91% (0.38 log reduction) which was significant difference ($P < 0.05$) from other fermented cereal extracts followed by fermented black glutinous rice extract (89.51% survival rate). However, there was no significant difference ($P > 0.05$) in survival rate of the strain in fermented Red kidney bean extract (87.74% survival rate) and White kidney bean

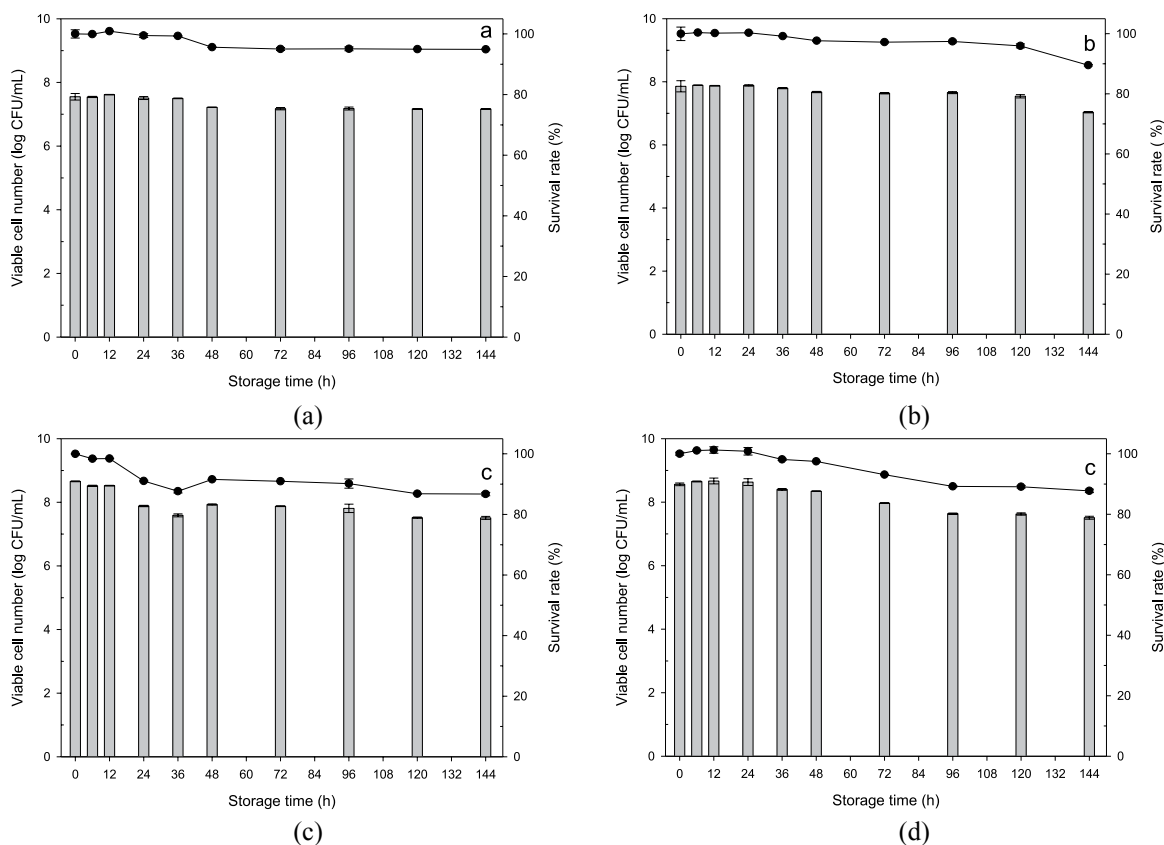


Figure 5: Viable cell number and survival rate of *L. plantarum* TISTR 2075 during storage at 4°C for 144 h in fermented Plai Ngahm Prachin Buri rice extract (a), fermented black glutinous rice extract (b), fermented White kidney bean extract (c), and fermented Red kidney bean extract (d).

Grey bar represents viable cell number (log CFU/mL) and —●— represents the survival rate (%). Values with different lowercase letter (a-c) of survival rate at storage time of 144 h are significantly different by Duncan's multiple range test ($P < 0.05$).

extract (86.73% survival rate) (Figure 5). The highest survival rate of *L. plantarum* TISTR 2075 stored at 37°C was detected in fermented black glutinous rice extract (83.40% survival rate and 1.3 log reduction). A significant difference ($P < 0.05$) in survival rate at the end of storage time was also observed. In this storage condition, the survival rate of the strain fermented in Plai Ngahm Prachin Buri rice extract was 76.90% which was not significant difference ($P > 0.05$) from the strain in fermented White kidney bean extract (76.54%). The strain fermented in Red kidney bean extract exhibited the lowest survival rate of 70.49% (Figure 6).

The stability of *L. plantarum* TISTR 2075 in fermented cereal extract kept under 4°C and 37°C could be considered in a term of the specific rate of

degradation (k , h^{-1}). As shown in Figure 7, it was obvious that the storage temperature was a crucial parameter affecting the survival of the strain. Higher storage temperatures led to a decrease in the number of viable bacteria [36,37]. At 4°C, the strain fermented in Plai Ngahm Prachin Buri rice extract exhibited the lowest k value of 0.0034 h^{-1} which was lower than fermented black glutinous rice extract, fermented Red kidney bean extract and fermented White kidney bean extract, respectively. The same tendency was also observed at non-refrigerated temperature of 37°C. The strain fermented in black glutinous rice extract showed the lowest k value of 0.0181 h^{-1} followed by fermented Plai Ngahm Prachin Buri rice extract, Red kidney bean extract and White kidney bean extract, respectively (Table 2).

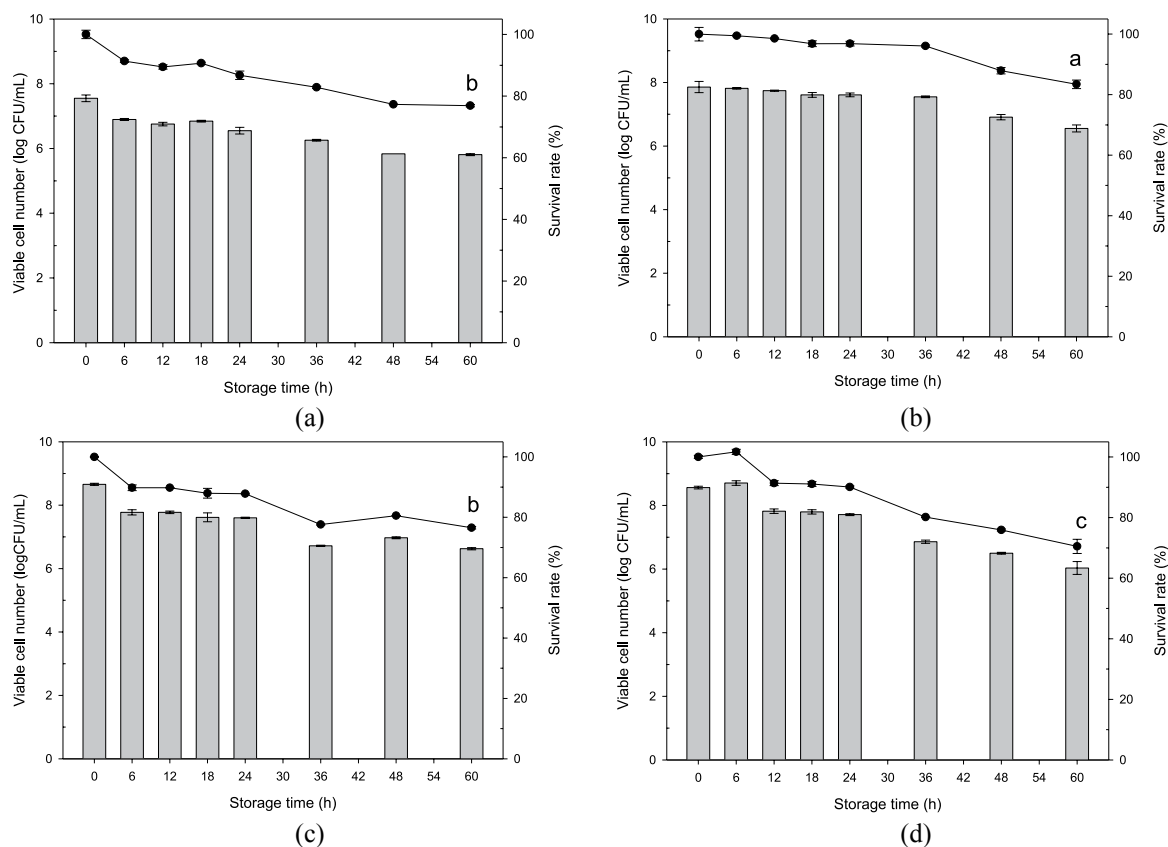


Figure 6: Viable cell number and survival rate of *L. plantarum* TISTR 2075 during storage at 37°C for 60 h in fermented Plai Ngahm Prachin Buri rice extract (a), fermented black glutinous rice extract (b), fermented White kidney bean extract (c), and fermented Red kidney bean extract (d).

Grey bar represents viable cell number (log CFU/mL) and —●— represents the survival rate (%). Values with different lowercase letter (a-c) of survival rate at storage time of 60 h are significantly different by Duncan’s multiple range test ($P < 0.05$).

Table 2: Specific rate of degradation (k , h^{-1}) of *L. plantarum* TISTR 2075 in fermented cereal extracts during storage at 4°C and 37°C

Fermented Cereal Extracts	Specific rate of degradation of <i>L. plantarum</i> TISTR 2075 kept at 4°C		Specific rate of degradation of <i>L. plantarum</i> TISTR 2075 kept at 37°C	
	k_4 (h^{-1}) ^a	R ^{2b}	k_{37} (h^{-1}) ^a	R ^{2b}
Fermented Plai Ngahm Prachin Buri rice extract	0.0034	0.773	0.0344	0.792
Fermented black glutinous rice extract	0.0039	0.770	0.0181	0.863
Fermented White kidney bean extract	0.0098	0.484	0.0401	0.646
Fermented Red kidney bean extract	0.0076	0.898	0.0426	0.957

^a The slope of the regression lines, as shown in Figure 7, were taken the inactivation rates.

^b R²: coefficient of determination

From results, the first order kinetic model was applied to fit a linear survival curve. However, the results revealed that coefficient of determination

(R²) of k_4 of the strain fermented in White kidney bean extract was not followed the linear regression model. Therefore, Gompertz equation: $\ln \frac{N}{N_0} = ae^{-e^{(b-t)}}$;

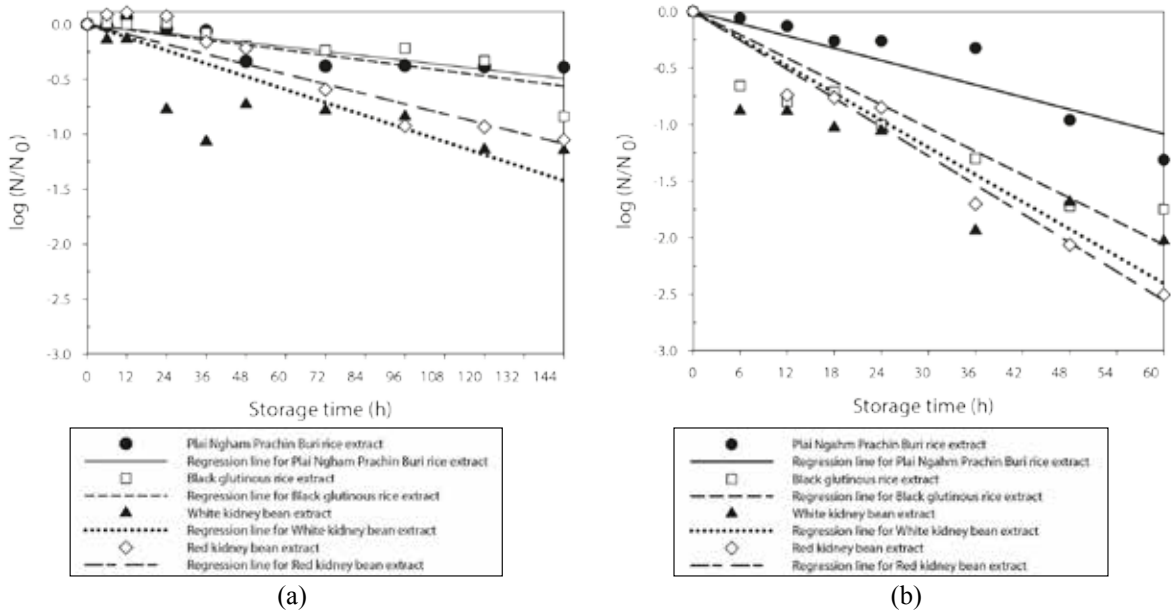


Figure 7: Storage stability of *L. plantarum* TISTR 2075 expressed as the logarithmic values of relative survival fraction $\log(N/N_0)$ against storage time at 4°C for 144 h (a), and 37°C for 60 h (b).

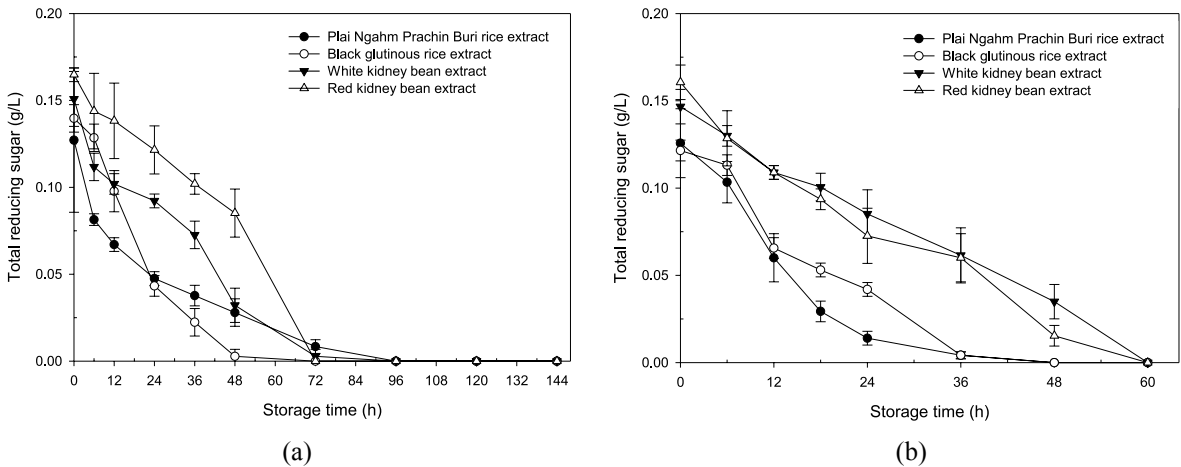


Figure 8: Total reducing sugar concentration of different fermented cereal extracts kept at 4°C for 144 h (a), and 37°C for 60 h (b).

where constants a , b and c are obtained by fitting experimental data [38-40], was introduced to fit this survival curve. From the results, a non-linear regression equation: $y = -2.159 \cdot \exp\{-e^{(2.869-0.187t)}\}$ and R^2 value of 0.925 was achieved from this proposed model. The inactivation rate constant defined as the slope of the tangent at the inflection point was found to be 0.033 h^{-1} . The Gompertz equation and its modified form has been used to fit non-linear survival curves

of *L. monocytogenes* Scott A [41]. This model has also been successfully used to describe growth and kinetics of *L. plantarum* in the fermentation of edible Irish brown seaweeds [38].

Results presented in Figure 8 showed TRS profiles of fermented cereal extracts kept under 4°C for 144 h and 37°C for 60 h. The evolution of TRS in all fermented cereal extracts exposed similar trends. TRS evolution of all fermented cereal extracts continuous

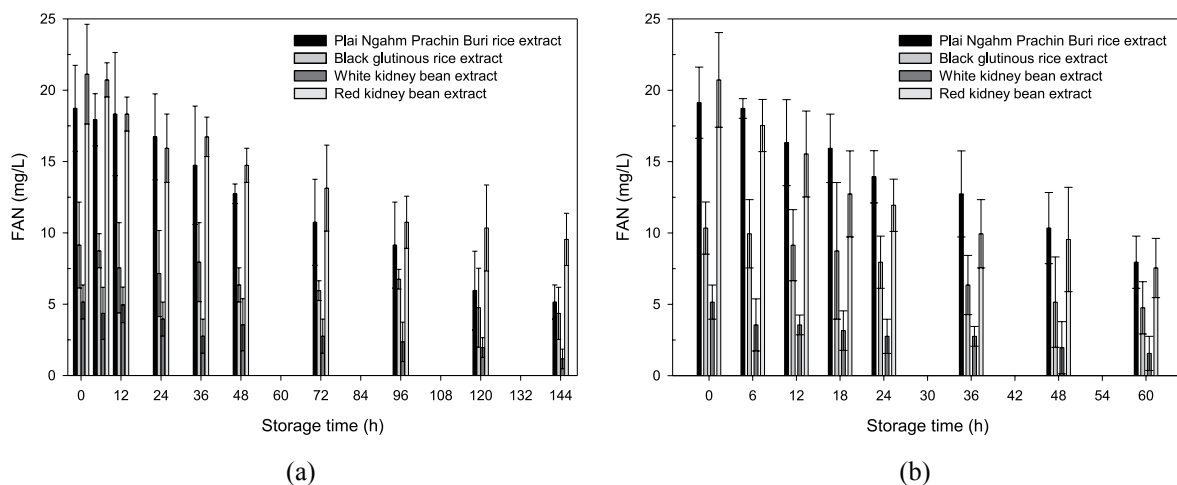


Figure 9: Free amino nitrogen (FAN) evolution of different fermented cereal extracts during storage at 4°C for 144 h (a), and 37°C for 60 h (b).

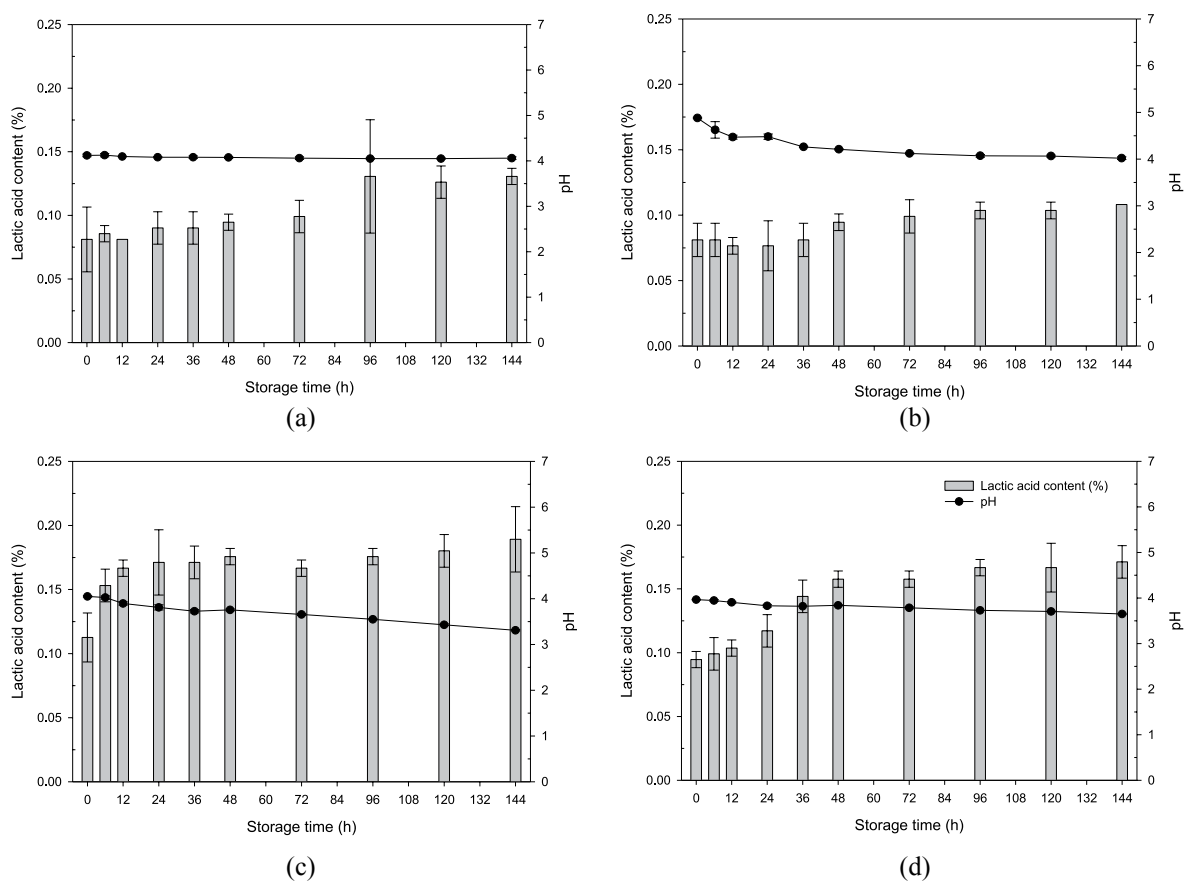


Figure 10: Change in pH and lactic acid content in fermented Plai Ngahm Prachin Buri rice extract (a), fermented black glutinous rice extract (b), fermented White kidney bean extract (c), and fermented Red kidney bean extract (d) during storage at 4°C for 144 h.

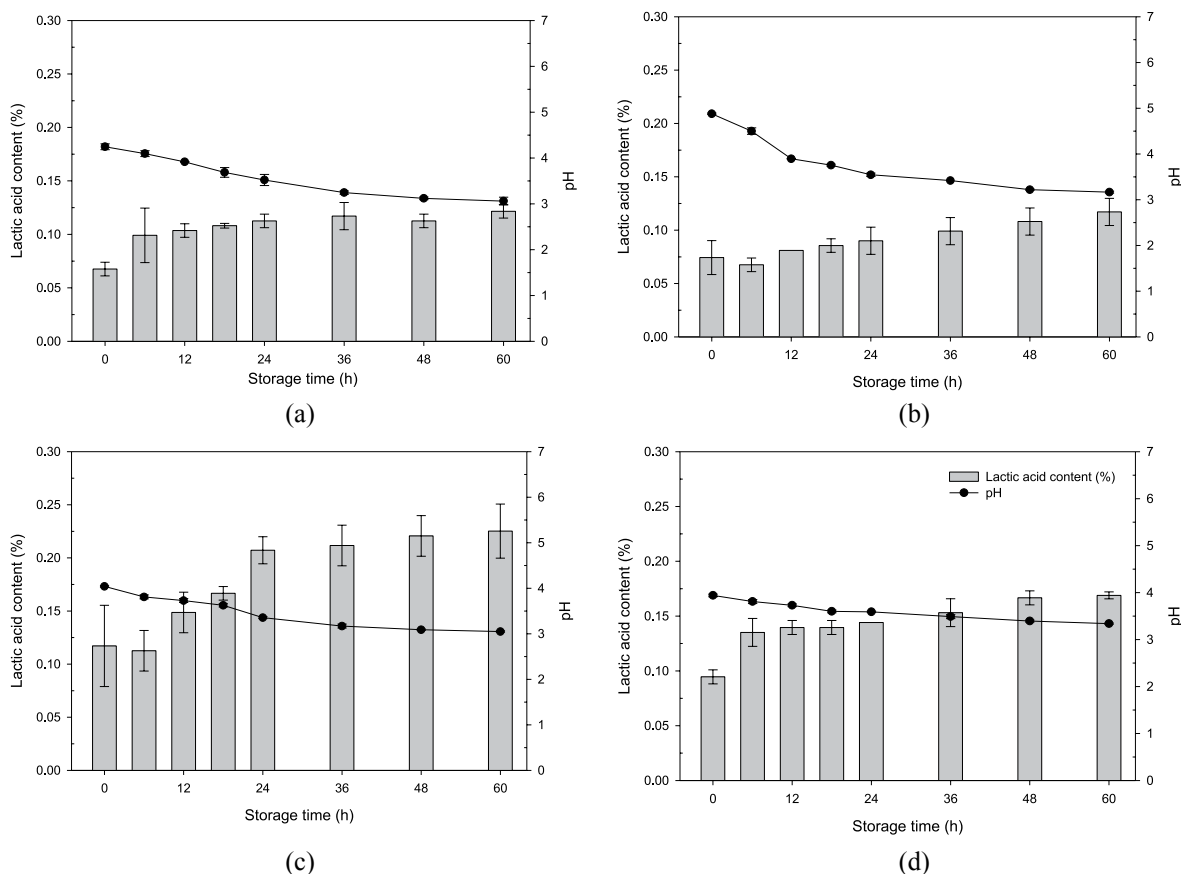


Figure 11: Change in pH and lactic acid content of fermented Plai Ngahm Prachin Buri rice extract (a), fermented black glutinous rice extract (b), fermented White kidney bean extract (c), and fermented Red kidney bean extract (d) during storage at 37°C for 60 h.

declined in storage period. The results revealed that TRS value in fermented black glutinous rice extract was found to decrease to undetectable level after storage for 48 h at 4°C and 37°C. At storage temperature of 4°C, TRS value of fermented Red kidney bean extract reached to undetectable value at 72 h while the same trends were observed in fermented Plai Ngahm Prachin Buri rice extract and White kidney bean extract at 96 h. However, a rapid decrease in TRS evolution was detected at 37°C. Fermentable sugars in cereal suspension provided the carbon components for the growth of microorganisms [25]. It is possible that the TRS increments could be due to the release of monosaccharides and disaccharides from the cereal starch due to the amylase activity within the lactic acid bacteria [16].

As shown on Figure 9, it was observed that the

values of FAN in fermented cereal extracts tended to decrease until the end of storage period. At 4°C, a slightly reduction in FAN value was noticed. Fermented Plai Ngahm Prachin Buri rice extract exhibited the highest reduction in FAN value of 13.57 mg/L. While, fermented Red kidney bean extract showed 13.17 mg/L reduction under storage at 37°C.

The profiles of lactic acid evolve in a different manner depending on the medium. Lactic acid contents produced from fermented cereal extracts by *L. plantarum* TISTR 2075 after storage at 4°C and 37°C were shown in Figure 10-11. The amount of lactic acid ranged from 0.11% to 0.19% and 0.12% to 0.23% in fermented cereal extracts kept under 4°C for 144 h and 37°C for 60 h, respectively. The results revealed that the strain could be able to produce acid even at refrigerated temperature. This was in accordance with Guo et al.

[42] that *L. casei* reduced the pH value of fermented milk to pH 4.60 after storage at refrigerated temperature for 28 days. Among all fermented cereal extracts tested, fermented White kidney bean extract showed the highest lactic acid content of 0.19% and 0.23% under storage at 4°C and 37°C, respectively. Moreover, Salmerón et al. [16] suggested that *L. plantarum* is facultatively heterofermentative producing lactic acid, acetic acid and CO₂ as main end-products [43]. The evolution of pH decrease could be attributed to the growth of bacteria and lactic acid production. The accumulation of acids during storage is responsible for the decrease in growth rate [44]. The final pH of products was 3.31-4.06 and 3.05-3.34 under storage temperature of 4°C and 37°C. Also, fermented White kidney bean extract exhibited the lowest pH at the end of storage period at both 4°C and 37°C. The significantly lower pH and faster reduction in pH during storage of fermented White kidney bean extract may adversely affect probiotic growth and viability. This was in agreement with Espinoza and Navarro [45] that the decrease in pH of the medium and accumulation of lactic acid, diacetyl, and acetaldehyde from growth and fermentation are the main factors for viability loss of probiotics added to milk. Furthermore, Passos et al. [46] also reported that the production of lactic acid and organic acids could inhibit microbial growth in their undissociated form, dissociated form or indirectly by releasing the proton (H⁺) in the medium.

4 Conclusions

In summary, the four types of cereal extract demonstrated their capability to support probiotic fermentation by *L. plantarum* TISTR 2075. During fermentation at 37°C for 24 h, the highest viable cell number was achieved from fermented White kidney bean extract. The storage stability of the strain in fermented cereal extracts could be considered in term of *k* value. It could be indicated that Plai Ngahm Prachin Buri rice extract displayed the highest storage stability with the lowest *k* value during storage at 4°C for 144 h. While, black glutinous rice extract exhibited the highest storage stability during storage at 37°C for 60 h. These results suggest that these cereal extracts could be used as culture media for the growth of probiotic *L. plantarum* TISTR 2075. This finding could further lead to the

development of novel probiotic beverage products. The consumer acceptability should be also evaluated.

Acknowledgements

The authors express their sincere appreciation to the Science and Technology Research Institute, King Mongkut's University of Technology North Bangkok for financially supporting.

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