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Use of Rice Hull Hydrolyzate in the Cultivation of *Lactobacillus acidophilus*

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Abstract

This project aims to minimize the cost of production of live *L. acidophilus* by replacing the expensive carbon source in the fermentation medium with the rice hull hydrolyzate and compare the growth efficiency of the microbe in the rice hull formulated medium with the rich medium (de Man Rogosa Sharpe or MRS). Rice hull was hydrolyzed with diluted H_2SO_4 and HCl at various concentrations 0.25, 0.5 and 1% v/v at 120°C for 30 minutes. The hydrolyzate was further overliming with $Ca(OH)_2$ and filter through the diatomaceous earth. The treatment with 0.5% and 1% HCl provided the reducing sugar of 23.05 and 27.45 g/L which were close to MRS broth of 20.88 g/L. The hydrolyzate from the treatment with 1% HCl was further mixed with MRS in the ratio of MRS: hydrolyzate of 25: 75 and 10: 90 v/v. The MRS mixed with the hydrolyzate in the ratio of 25:75 v/v gave the maximum specific growth rate (μ_{max}) of 0.437 $hour^{-1}$ which was comparable to the MRS broth of 0.424 $hour^{-1}$. The biomass yield ($Y_{x/s}$), dry weight and viable cell number on the formulated medium at 48 hours cultivation were 0.21 g/g, 2.61 mg/ml and 2.07×10^8 CFU/ml respectively in comparison to MRS at 48 hours cultivation which were 0.19 g/g, 3.07 mg/ml and 2.19×10^8 CFU/ml (viable cell number in MRS represented the maximum level at 9 hours) respectively. The total acidity produced by the culture in formulated medium and MRS were 1.461% and 1.761%. Although, the formulated medium from rice hull hydrolyzate reduced the production cost in term of medium requirement, the time required for obtaining the same amount of viable cells in the hydrolyzate was longer than using MRS as a sole medium.

Keywords: *Lactobacillus acidophilus*, Lignocellulosic waste, Rice hull, Hydrolyzate

1. Introduction

Lignocellulosic materials have been considered as abundant wastes in the world. They are cheap renewable resources that are available in large quantities. As Thailand is a nation's leading producer in rice, million tonnnes of the rice hull have been left as one of the abundant lignocellulosic wastes in Thailand after the rice paddy. Rice hull composes of lignin 16%, cellulose 36% and hemicelluloses 12% so, it is a potential source of fermentable carbohydrates that remains under utilization (1). In order to make the structure of lignocellulosic materials to be accessible by the fermentable microbes, the polymers like cellulose and hemicelluloses must be hydrolyzed to be sugar monomers. Normally, the pretreatment process will be processed through the physical and chemical methods prior the enzymatic hydrolysis (2). Acid hydrolysis of lignocelluloses is commonly conducted by diluted H_2SO_4 or HCl in the range of 2-5% at the temperature around 160^{o}C (3). However, the diluted acid treatment at higher temperature and pressure develops many toxic compounds that stress the fermentative organisms. Therefore, there are numerous of detoxification methods including biological, physical and chemical. One of the cost effective detoxification method is the use of $Ca(OH)_2$ for an overliming process (4). The toxic compounds are also potentially removed by the diatomaceous earth (5). These two chemical methods are commonly used to remove toxic compound from lignocellulosic hydrolyzates. The hydrolyzates from lignocelluloses have been widely used as potential carbon sources for many fermentation processes for the microbial cultivations and production of value-added products (1,6).

Probiotics have been widely used as animal feed supplement that are the live microbes represent beneficially affect on the health of the host animal upon the ingestion by improving the intestinal microbial balance (7). The probiotics that exist in the market are commonly made from the viable lactic acid bacteria. *Lactobacillus acidophilus* has been used as a live probiotics to mix with animal feed in order to promote the growth and prevent intestinal infections (8,9). Mostly, *Lactobacillus* sp. requires complex nutrients and carbon sources for cell growth and development which causes the escalation in the cost of the feed supplement. Therefore, lignocellulosic hydrolyzates have been emerged as alternative carbon source to substitute the used of expensive carbon source in the cultivation. Lignocellulosic hydrolyzates have been successfully used for the cultivation of *Lactobacillus* sp. and the lactic acid production. *Lactobacillus* sp. represent the abilities to transform a wide range of carbohydrates from the lignocellulosic hydrolyzates for cell growths and lactic acid production such as corn cob hydrolyzate (10). Therefore, this research aims to use the rice hull hydrolyzate as a low cost carbon source to minimize the use of rich medium or substitute the glucose utilization or other monosaccharides for *L. acidophilus* culture. The growth efficiencies of the microbes in the rice hull formulated medium and the rich medium (de Man Rogosa Sharpe or MRS) were compared based on maximum specific growth rate, biomass yield, dry weight, viable cell number and percent acidity.

2. Materials and Methods

2.1 Bacterial strain and cultivation medium

L. acidophilus train TISTR 1138 was obtained from Thailand Institute of Scientific and Technological Research (TISTR). The culture was maintained in -20^{o}C frozen storage. Prior the use, the culture was grown on de Man Rogosa Sharpe or MRS agar (in the unit of g/L composes of peptone 10.0; meat extract 8.0; yeast extract 4.0; D-glucose 20.0; dipotassium hydrogen phosphate 2.0; Tween® 80 1.0; di-ammonium hydrogen citrate 2.0; sodium acetate 5.0; magnesium sulfate 0.2; manganese

sulfate 0.04 and agar 15) and transferred to MRS broth at 37°C for 24 hours and subculture once. The final overnight culture with the optical density (OD_{600}) of approximately 2.0 was used for the study.

2.2 Pretreatment of rice hull

Milled rice hulls in the total of 10% w/v were slurried in diluted H_2SO_4 and HCl at the various concentrations of 0.25%, 0.5% and 1% v/v and pretreatment in the autoclave at 120°C for 30 minutes. The pretreated hydrolyzates were filtered and hydrolyzate was further overliming with $Ca(OH)_2$ to pH 10 and adjusted with HCl to pH 4.5. The hydrolyzates were further filtered through diatomaceous earth. The hydrolyzates were placed in the autoclave to sterile at 121°C for 20 minutes. The amount of reducing sugar from each hydrolyzate under each treatment was analyzed by dinitrosalicylic acid (DNS) method using glucose as a standard (11). The amount of reducing sugar from each hydrolyzate was compared with MRS broth. The assay was performed triplicate and the mean value was identified.

2.3 Determination on the growth effect of each rice hull hydrolyzate

The optimum pretreatment processes were chosen based on the top three treatments that provided the highest concentration of reducing sugar from the previous method. The MRS broth was mixed with the hydrolyzate from each optimum pretreatment in the ratio of 25: 75 v/v. The maximum specific growth rate (μ_{max}), dry weight (mg/ml) and reducing sugar consumed (mg/ml) from each condition was identified. All experimental assays were performed triplicate. The condition that provided the highest in maximum specific growth rate, dry weight and reducing sugar consumption which were comparable to the MRS was chosen to perform the next experimental assay.

2.4 Formulated rice hull hydrolyzate

The rice hull hydrolyzate (RH) from optimum pretreatment process was formulated with MRS and MRS without glucose (modified MRS) in the different ratio in order to minimize the usage of the MRS broth and compare the effectiveness of each mixture over the bacterial growth. The optimum formulated medium will be the one in which obtaining the similar growth kinetics and percent acidity in comparable to the culture in MRS broth. The mixtures were set as follow; MRS broth with distilled water in the ratio of 10: 90 v/v, MRS broth with distilled water in the ratio of 25: 75 v/v, MRS broth with RH in the ratio of 10: 90 v/v, MRS broth with RH in the ratio of 25: 75 v/v, modified MRS broth with RH in the ratio of 25: 75 v/v, modified MRS broth with RH in the ratio of 10: 90 v/v, modified MRS 100%, RH hydrolyzate 100% and MRS 100%. All of the mixtures were prepared in the volume of 50 ml, adjusted pH to 4.5 and sterilized by autoclaving at 121°C for 20 minutes. The total of 2% v/v of overnight *L. acidophilus* culture (OD_{600} approximately 2.0) was inoculated into each condition. The maximum specific growth rate (μ_{max}), biomass yield ($Y_{x/s}$), dry weight (mg/ml), viable cell number (CFU/ml) and total acidity were measured in all mixtures. The maximum specific growth rate was analyzed based on the OD_{600}. The cell dry weight was measured based on the weight of the dried cell. The viable cell number was measured by plating the proper diluted culture on MRS agar plate. The percent acidity was used as a parameter to measure on the cell metabolism. The percent acidity was measured using the titration method with 0.1N NaOH and phenolphthalein as an indicator. All experimental assays were performed triplicate and the data was shown in the mean value.

2.5 Statistical analysis

The mean values of all replication were calculated using excel software. The differences were identified based on the P-values from unpaired t-test compare with MRS.

3. Result and Discussion

Rice hull is an abundant agricultural waste in Thailand. The purpose of this study was to study on the potential of using rice hull hydrolyzate for the cultivation of *L. acidophilus* in order to replace the expensive carbon source. The rice hull formulated medium was developed to compare on the bacterial growth efficiency with the rich medium (de Man Rogosa Sharpe or MRS). The pretreatment of rice hull is crucial. Dilute acid pretreatment has been widely used for pretreating any lignocellulosic material (1,12,13). Therefore, the milled rice hulls in this study were initially pretreated with diluted H_2SO_4 and HCl at the various concentrations of 0.25%, 0.5% and 1% v/v at 120°C in the autoclave and followed by overliming and filtered through the diatomaceous earth. The amount of reducing sugar from each treatment was analyzed by DNS method using glucose as a standard. The reducing sugar increases accordingly to the increase in the concentration of the acid on the pretreatment process (table 1). The treatment with 0.5% and 1% HCl provided the reducing sugar in the rate of 23.05 and 27.45 g/L respectively which were even higher than the MRS broth of 20.88 g/L. The P-value of the reducing sugars of all pretreatment processes were shown to be less than 0.0001 where the differences were considered to be extremely statistically significant. The pretreatment processes in which had shown to be the optimum pretreatment processes were chosen based on the amount of the reducing sugars to initially determine on the effect of the hydrolyzate over the growth of *L. acidophilus*. The MRS broth was mixed with the hydrolyzate from each optimum pretreatment in the ratio of 25: 75 v/v. The maximum specific growth rate (μ_{max}, $hour^{-1}$) and dry weight (mg/ml) and amount of reducing sugar consumed (mg/ml) were investigated to compare with the MRS broth. From the result (table 2), the μ_{max} of RH from 1% HCl pretreatment provided the P-value of non-significant different from the μ_{max} of MRS, where there were 0.361 $hour^{-1}$ in the 1% HCl treatment and 0.408 $hour^{-1}$ in the MRS broth. In addition, the 1% HCl pretreated hydrolyzate also provided the highest dry weight and the highest reducing sugar consumption rate than other treatments in which the values were 2.55 mg/ml and 11.11 mg/ml respectively. The obtained dry weight and reducing sugar consumption rate of 1% HCl treatment were approximately the same to the MRS broth that were 2.89 mg/ml and 16 mg/ml respectively. The pretreatment of rice hull with 1% HCl was considered to provide the less growth effect over the growth of *L. acidophilus* with the high metabolic rate in comparison to the MRS. Therefore, the RH from 1% HCl pretreatment was chosen to perform the medium formulated assay.

Table 1. The amount of reducing sugar obtained from rice hull under each specific pretreatment method was analyzed using DNS method (glucose as a standard)

Pretreatment processes	Concentration of reducing sugar (g/L)	P-value of reducing sugar
Heated with 0.25 % H_2SO_4	5.41 ± 0.021	<0.0001
Heated with 0.50 % H_2SO_4	8.71 ± 0.017	<0.0001
Heated with 1.00 % H_2SO_4	16.40 ± 0.021	<0.0001
Heated with 0.25 % HCl	18.50 ± 0.020	<0.0001
Heated with 0.50 % HCl	23.05 ± 0.020	<0.0001
Heated with 1.00 % HCl	27.45 ± 0.021	<0.0001
MRS (control)	20.88 ± 0.021	1

Table 2. The table represents the effects of the rice hull hydrolyzates from the different pretreatments over the growth of *L. acidophilus*. The analysis was based on the maximum specific growth rate ($hour^{-1}$), dry weight (mg/ml) and the amount of reducing sugar consumed (mg/ml). One asterick means the P-value was less than 0.05 and two asterick means the P-value was less than 0.0001 that were compare to the MRS broth.

25:75 MRS: RH of each specific pretreatment	Specific growth rate ($hour^{-1}$)	Dry weight (mg/ml)	Reducing sugar consumed (mg/ml)
0.25 % HCl	0.347*	1.48*	4.45**
0.5 % HCl	0.382	2.21*	4.31**
1 % HCl	0.361	2.55*	11.11**
MRS (control)	0.408	2.89	16.0

In order to meet the project aims to minimize the cost of production of live *L. acidophilus*, RH was formulated to replace the usage of the rich medium (MRS). The effectiveness of each mixture over the growth of bacteria was compared to the MRS broth based on the maximum specific growth rate, dry weight, viable cell number and total acidity. The MRS mixed with the hydrolyzate in the ratio of 25: 75 v/v gave the maximum specific growth rate of 0.437 $hour^{-1}$ which was approximately the same to the MRS broth of 0.424 $hour^{-1}$. The P-values of these 2 conditions were calculated and the values were considered to be not statistically significant difference while with other conditions were found to be significant differences with the P-values of less than 0.05. Thus, the medium formulated with 25:75 MRS: RH provided the similar effectiveness over the growth of bacteria to MRS broth. The reduction in the ratio of MRS to RH and the modified MRS without glucose mixed with RH represented the dramatic effect on the growth of *L. acidophilus*. The biomass yield (Yx/s), dry weight on the formulated medium with 25: 75 MRS: RH at 48 hours cultivation were 0.21 g/g and 2.61 mg/ml respectively which were comparable to MRS of 0.19 g/g and 3.07 mg/ml. The maximum viable cell number was found to be 2.19×10^8 CFU/ml on MRS at 9 hours cultivation while it was 2.07×10^8 CFU/ml on

the formulated medium at 48 hours cultivation (table 4). The rice hull hydrolyzate from diluted acid pretreatment and enzymatic saccharification was found to compose of fermentable sugar like glucose, xylose, arabinose, galactose (Saha et al., 2005). The diluted acid pretreatment by 1% HCl was only used to hydrolyze the rice hull in order to reduce the need for enzyme hydrolysis. However, the process was possibly released inhibitory compounds during the pretreatment even the overliming and filtration through the diatomaceous earth had been processed. Therefore, the formulated medium with rice hull hydrolyzate required a longer time to produce the equivalent amount of viable cell than the MRS (figure 1, table 4). The total acidities from MRS: hydrolyzate 25: 75 and MRS were produced in proportional to the bacterial growth where they had shown to be 1.461% and 1.761% at 48 hours respectively. Although, the mixture of rice hull hydrolyzate could be used to substitute the usage of MRS, the cultivation time requirement to obtain the same amount of viable cell number was longer than using MRS as a sole medium. The only rice hull hydrolyzate or even the rice hull hydrolyzate with modified MRS illustrated the significant reduction on the bacteria growth. The reduction in the ratio of MRS to the hydrolyzate also reduced the growth efficiency of *L. acidophilus*. Therefore, the rice hull hydrolyzate represents a potential to be used to substitute the expensive carbon source or expensive medium as MRS in the proper formulated.

Table 3. Effect of formulated medium over the growth of *L. acidophilus* based on the maximum specific growth rate (μ_{max}, $hour^{-1}$) and dry weight (mg/ml). The P-value of each condition was compared to MRS.

Type of medium	μ_{max} ($hour^{-1}$)	P-value of Specific growth rate	Dry weight (mg/ml)	P-value of dried weight
10: 90 MRS: water	0.311	0.0015	0.767	<0.0001
25: 75 MRS: water	0.332	0.0103	1.15	<0.0001
10:90 MRS: RH	0.354	0.0180	1.85	0.0019
25:75 MRS:RH	0.437	0.713	2.61	0.0309
25:75 modified MRS: RH	0.321	0.0029	1.96	0.0272
10: 90 modified MRS: RH	0.182	<0.0001	1.51	0.0003
Modified MRS	0.248	<0.0001	0.373	<0.0001
MRS	0.424	1	3.07	1
RH only	0.217	0.0003	0.44	<0.0001

Table 4. The viable cell number (CFU/ml) in each condition after 0, 9, 24, and 48 hour(s) incubation time

Type of medium	0 hour CFU/ml	9 hours CFU/ml	24 hour CFU/ml	48 hour CFU/ml
10:90 MRS : water	1.01×10^{6}	1.92×10^{7}	1.10×10^{7}	1.02×10^{7}
25:75 MRS : water	1.01×10^{6}	8.35×10^{7}	4.13×10^{7}	2.30×10^{7}
10:90 MRS : RH	1.03×10^{6}	5.88×10^{7}	8.47×10^{7}	1.34×10^{8}
25:75 MRS : RH	1.13×10^{6}	4.32×10^{7}	1.34×10^{8}	2.07×10^{8}
RH only	9.20×10^{5}	4.17×10^{6}	3.00×10^{6}	1.22×10^{7}
25:75 modified MRS: RH	1.04×10^{6}	4.62×10^{7}	7.23×10^{7}	1.56×10^{8}
10:90 modified MRS : RH	1.07×10^{6}	8.95×10^{7}	1.08×10^{8}	1.16×10^{8}
Modified MRS	1.21×10^{6}	1.37×10^{7}	1.05×10^{7}	8.50×10^{6}
MRS	1.13×10^{6}	2.19×10^{8}	9.55×10^{7}	8.17×10^{7}

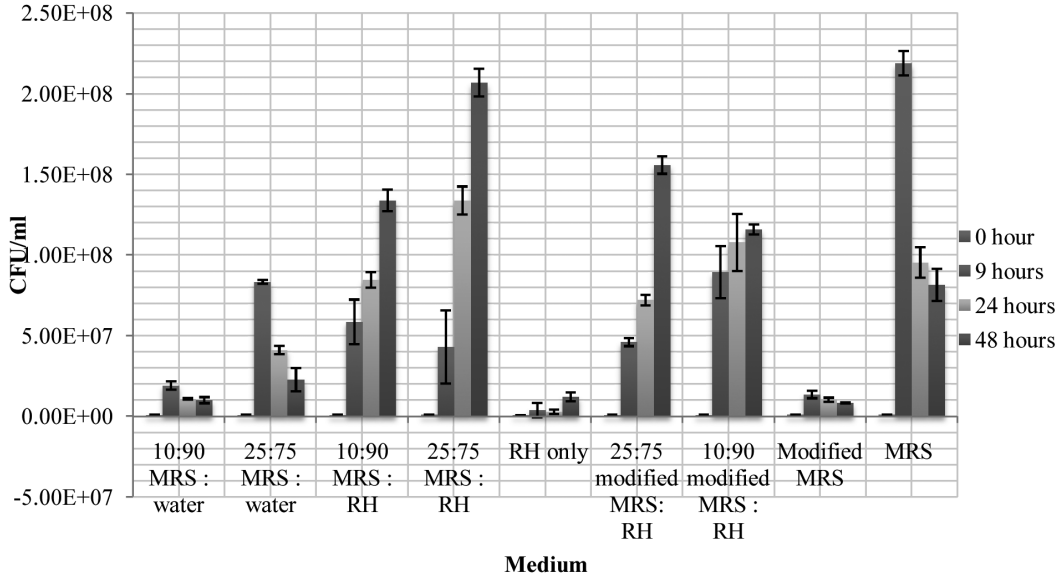

Figure 1. The viable cell number (CFU/ml) in each type of medium after 0, 9, 24, and 48 hour(s) incubation time

Table 5. Percentage of total acidity in each condition at 4 time points 0, 9, 24 and 48 hour (s)

Type of medium	0 hour	9 hours	24 hour	48 hour
10:90 MRS : water	0.015	0.143	0.161	0.153
25:75 MRS : water	0.064	0.534	0.636	0.648
10:90 MRS : RH	0.084	0.396	0.576	0.888
25:75 MRS : RH	0.072	0.267	0.882	1.461
RH only	0.065	0.096	0.282	0.309
25:75 modified MRS: RH	0.093	0.311	0.504	1.098
10:90 modified MRS : RH	0.095	0.824	0.816	0.900
Modified MRS	0.185	0.131	0.125	0.132
MRS	0.032	1.347	1.647	1.761

4. Conclusions

In conclusion, the mixture of rice hull hydrolyzate with MRS in the ratio of 75:25 v/v was effectively used for the cultivation of *L. acidophilus* by providing the similar growth kinetics to the pure MRS broth. However, the viable cell number appeared to be approximately the same in the later stage. This probably caused by the present of inhibitors from the pretreatment process that perturbed the bacterial growth. Therefore, the rice hull has a potential to replace the expensive carbon sources or expensive media for the cultivation of *L. acidophilus* or even application to other microorganisms. However, the further attempts probably focus on the pretreatment and saccharification process of rice hull to increase the reducing sugar yield with the reduction of inhibitors in which it will lead to improve the bacterial growth.

5. References

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