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An optimized sustainable medium for the direct lactic acid fermentation of sago (*Metroxylon sagu* Rottb.) starch by *Enterococcus faecium* DMF78

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Abstract

A low-cost medium for the amylolytic lactic acid bacterium, *Enterococcus faecium* DMF78, was developed by response surface methodology using sago starch as substrate. Six nitrogen sources, wort, whey, corn steep liquor (CSL), trub, soybean and mungbean flour were evaluated to completely eliminate or dramatically reduce the use of expensive yeast extract, beef extract, and proteose peptone in the medium. Highest lactic acid yield was achieved using a medium with 35.0% whey, 25.0% wort, 3.0% diluted CSL and 0.076% proteose peptone in the standard formulation of de Mann, Rogosa and Sharpe (MRS) medium. Validation run of the optimized medium in the 2-L fermentor revealed slightly lower starch utilization (0.75 g/g) and low cell growth, but high lactic acid yield of 28.83 g/L after 24 hours, as compared to fermentation using complete sago-MRS medium.

Introduction

Lactic acid is used in the food industry as flavor agent, preservative and acidifier. It is also used in other industries that manufacture leather, textile, pharmaceuticals, pesticides, herbicides and cosmetics (Vickroy, 1985). The estimated world demand for lactic acid is about 130,000-150,000 metric tons per year (Mirasol, 1999) and demand continues to rise because of the increasing demand for green or Earth-friendly materials. Lactic acid is the monomer for the production of polylactic acid (PLA), which is a bioplastic material that can be used to manufacture disposable kitchen wares and even high-end biomedical products. PLA is used in making biodegradable implants to repair fractures and injuries such as broken bones (Vickroy, 1985; Datta *et al.*, 1995). Because of its biocompatibility, PLA has been approved by the US FDA in 2004 for the treatment of facial fat loss, to thicken skin and improve the appearance of folds and sunken areas.

Lactic acid may be produced chemically or through microbial fermentation (Wee *et al.*, 2006). While chemical synthesis produces racemic mixtures, some microorganisms can selectively produce pure optical isomers (Pandey *et al.*, 2001). This gives fermentation an advantage over chemical synthesis, as it eliminates the costly process of enantiomeric separation, when pure isomers are needed, especially for the synthesis of high quality PLA. However, most lactic acid bacteria are not able to assimilate more complex carbon sources like starches and dextrins and rely on simple sugars, like glucose, for their metabolism. In addition most lactic acid bacteria optimally grow on a rather costly medium called de Mann, Rogosa and Sharpe (MRS) medium which contain proteose peptone, yeast extract and beef extract that play important roles as nitrogen and vitamin sources (Tejayadi and Cheryan, 1995; Altaf *et al.*, 2005).

An amylolytic homofermentative lactic acid bacterium *Enterococcus faecium* DMF78 isolated and characterized by Dr. Dulce M. Flores from puto (rice cake) fermentation starter was shown to grow optimally on MRS medium where the sole carbon source is sago starch (Shibata *et al.*, 2007). The amylolytic property of *E. faecium* DMF78 eliminated the use of more expensive fermentation feedstock

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such as glucose or the energy-extensive and enzyme-intensive steps of starch saccharification and liquefaction. While direct fermentation is definitely an added advantage, expensive nitrogen sources in the fermentation medium are deterrent in bringing the process to commercial scale production.

In the present study, we report a sustainable medium where the primary carbon source is sago starch with low-cost nitrogen sources optimized by response surface methodology using Box-Behnken design of experiment (Box and Behnken, 1960). In order to increase fermentation to a larger-scale, a medium with components derived from industrial by-products would be more viable. Studies made on alternative large scale fermentation media for lactic acid fermentation were made primarily to reduce costly nitrogen sources. These include the use of corn steep liquor or CSL (Wee *et al*., 2008), whey (Wee *et al.*, 2006), trub or lees (Bustos *et al*., 2004), soybean flour (Altaf *et al*., 2005), tofu liquid waste (Yuwono and Hadi, 2008) and others. However, to our knowledge, no report has been made on the possibility of mixing cost effective nitrogen sources, to come up with the right balance to optimize lactic acid production. A full factorial design of experiment is quite impractical due to the number of possible combinations especially when many components will be considered. In this study, a total of ten factors at three levels are considered, a full factorial design would entail 59,049 runs. Thus, an incomplete factorial design called Box-Behnken design was employed, where only a total of 170 runs were needed to achieve the same end.

The Box-Behnken designs are a class of response surface designs that are useful in the same setting as the central composite design. This class of design avoids treatment combinations that are beyond the experimental boundaries because it does not include corner points, and star points. Moreover, these designs fit a quadratic model and the estimation variance does not vary too much inside the smallest hypercube containing the experimental points (Myers and Montgomery, 1995)

Materials and Methods

Chemicals and raw materials

Sago starch, soybean flour, mungbean flour and whey were obtained from the Department of Food Science and Chemistry, University of the Philippines Mindanao. Wort and trub were obtained from San Miguel Corporation (Darong, Davao del Sur), and CSL from Julu Enterprises (Toril, Davao City). All reagents were analytical grade or HPLC-grade.

Microorganism

E. faecium DMF78 (BIOTECH 10375) was maintained in sago-MRS medium i.e., the primary carbon source was sago.

Shake flask fermentation optimization and design of experiment

Initial screening was done to determine the optimum level for each candidate nitrogen source that can sustain the lactic acid fermentation of *E. faecium* DMF78. Wort, CSL, whey, soybean flour, mungbean flour, and trub substituted for proteose peptone, yeast extract, and beef extract in the MRS medium. The liquid components wort, trub, and whey were supplemented at 25%, 50% and 75% (v/v). Solid components mungbean and soybean flours were added at 1%, 3% and 5% (w/v). However, CSL was determined to have significant initial lactic acid content, and was therefore used in minimal amounts at 1%, 3% and 5% (v/v). The various media were dispensed in 125-mL Erlenmeyer flasks in three replicates, each containing 30 mL medium.

In the second screening, a total of 170 combinations were formulated according to the Box-Behnken design of experiment generated using the software MINITAB v16. A total of 10 factors were evaluated at three levels for each factor, (Table 1). Salts and tween-80 amounts were the same as that found in a typical MRS medium. In all these formulations, the main carbon source was 1% (w/v) sago starch. Media were sterilized in an autoclave at 15 psi, 121°C for 15mins and 1% (w/v) sago-MRS was used as control for all experiments.

Statistical analysis and response surface methodology

The software Minitab v16 was used to design the experiment and analyze the results. For the response surface methodology, a 10-factor Box-Behnken design was generated and augmented with 10 center points as the experimental set-up for the subsequent analysis. Initially, the full quadratic model was fitted to the experimental data using the coded levels. The general form of the full quadratic model includes all linear, square, and interaction terms using the following equation:

$$Y = b_o + \sum_{i=1}^{q} b_i X_i + \sum_{j=1}^{q} \sum_{i=1}^{q} b_{ij} X_i X_j \qquad (1)$$

In Equation (1), Y is the response corresponding to lactic acid yield. X_i to X_q are the explanatory or independent variables, b_o is the offset term, b_i are the estimated regression coefficients for the main effects and they are interpreted as the average change in the response for each unit change of the variable associated with that coefficient while maintaining the

Table 1. Independent variables for the Box-Behnken design of experiment

Variable	Coded levels		
	-1	0	1
Wort, % (v/v)	0	12.5	25
Whey, % (v/v)	0	12.5	25
CSL, % (v/v)	0	1.5	3
Trub, % (v/v)	0	1.5	3
Mungbean Flour, % (w/v)	0	0.5	1
Soybean Flour, % (w/v)	0	0.5	1
Yeast Extract, % (w/v)	0	0.025	0.05
Proteose Peptone, % (w/v)	0	0.05	0.1
Beef Extract, % (w/v)	0	0.05	0.1
Shaking speed, rpm	0	100	200

rest of the variables constant.

Two-liter fermentor validation runs

The medium optimized from a mathematical model based on the Box-Behnken design of experiment was validated using a 2-L fermentor (Labo-controller MDL-8C, B.E. Marubishi, Tokyo, Japan). Fermentation was carried out with 10% inoculum at 30°C without agitation, initial pH of 6.5, with a working volume of 1L. The pH of the culture was controlled by the addition of either 4M NaOH or 4 M HCl. Samples for analysis were immediately boiled for 10 mins to stop the fermentation.

Analytical methods

The amount of lactic acid produced was determined using HPLC (Shimadzu, Kyoto, Japan), equipped with SUPELCO Gel C-610H column and RID-10A detector (Shimadzu, Kyoto, Japan); 5mM H2SO4 was used as solvent at a flow rate of 1.0 mL/min; column oven was set at 50°C. Total sugars and residual starch were determined using anthrone method as described by McReady *et al.* (1950). Total reducing sugars was analyzed according to the DNS method by Borel *et al.* (1952). For viable cell count, a 1.0 mL appropriately diluted sample was pour-plated onto MRS agar with sago starch as carbon source. The plates were incubated at 30°C for 24 hours.

Results and Discussion

Initial screening for nitrogen alternatives

Initial lactic acid content of each chosen alternative nitrogen source was analyzed to be used as correction factor of the lactic acid yield to ensure that the lactic acid produced during fermentation can be attributed solely to the metabolic activity of *E. faecium* DMF78. Results show that whey, trub and CSL contain significant amounts of lactic acid at 2.24, 7.25 and 28.19 g/L, respectively. These were therefore used minimally to avoid significant lactic acid contamination.

Analysis of the various media formulations in the initial screening phase showed that 75% wort

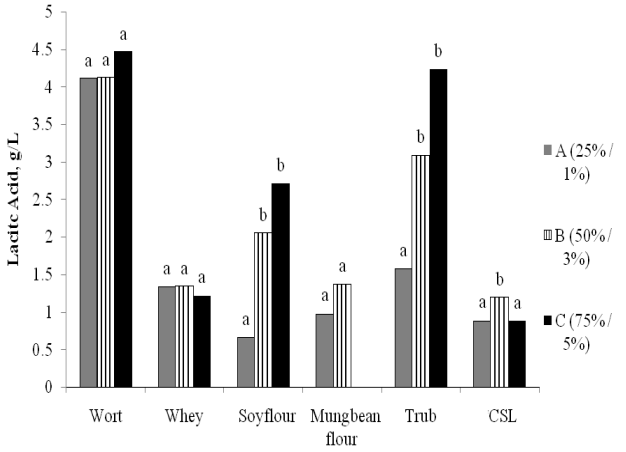

Figure 1. Net lactic acid production of the six alternative nitrogen sources after 24 hours of fermentation. (Bars with the same letter in each alternative media indicate insignificant difference at 95% level of confidence)

substitution gave the highest LA yield at 4.458 ±0.12g/L after 24 hours of fermentation, whereas sago-MRS control only produced 3.605±0.40g/L lactic acid. Further, ANOVA (p-value < 0.05) showed that there was no significant difference in lactic acid yield among the three wort levels used. Wort mainly used in beer making, is mashed malted barley containing sugars, soluble protein, polypeptides, amino acids, nitrogenous compounds, sulfur compounds and trace elements, with total nitrogen content of 0.88g/L (Briggs *et al*., 1981; Stewart, 2006). These nutrients seemed to support the metabolic needs of even this highly fastidious microorganism, *E. faecium* DMF78. The low lactic acid production (Figure 1) of the other alternatives may be due to unavailability of certain nutrients (vitamins and amino acids) as required by the microorganism (Altaf *et al*., 2005).

Mathematical modeling and response surface methodology

From the initial screening, it was shown that the selected nitrogen sources can sustain the lactic acid fermentation of *E. faecium* DMF78 albeit on varying degrees. The results of the experiments showed that the lactic acid yield values ranged from a minimum of 0.3170 g/L to a maximum of 6.617 g/L. This is indicative that lactic acid yield is strongly affected by

Table 2. Analysis of Variance for Lactic Acid Yield (g/L)

Source	DF	Seq SS	Adj SS	Adj MS	F-value	P-value
Regression	65	297.519	297.519	4.577	7.31	0.000
Linear	10	228.516	228.516	22.852	36.51	0.000
Square	10	23.653	23.653	2.365	3.78	0.000
Interaction	45	45.349	45.349	1.008	1.61	0.025
Residual Error	104	65.087	65.087	.626		
Lack-of-fit	95	60.112	60.112	.633	1.14	0.448
Pure Error	9	4.976	4.976	.553		
Total	169	362.606				

the variables selected for the study. This observation is further supported by the wide range of values taken by the coefficients of the variables in the model. The data was fitted to a quadratic model and regression analysis was done. The computed adjusted coefficient of determination (R^2) for the full quadratic model is 70.83%, which indicated a good fit. The model contained a total of 66 terms: 1 intercept, 10 linear, 10 square, and 45 interaction terms. Analysis of variance (ANOVA) of the quadratic regression model demonstrates that it is significant as indicated by its computed F-value (7.31), Table 2. The non-significance of lack-of-fit and the significance of square and interaction all indicate that a second-order polynomial is adequate to model the relationship between the response and the chosen explanatory variables. However, if the p-value of each of the term is examined, it can be seen that many of the terms in the model are not significant, i.e., some variables or interaction between variables do not contribute much to the variation in the response. Hence, it would be desirable to eliminate the non-significant terms in order to simplify the model and obtain a better fit.

Term selection was performed to retain only those terms that explain most of the variability in the response. The term selection proceeded as follows: those interaction terms and square terms that have p-values less than 0.05 were removed, and linear terms were retained if its p-value was less than 0.05 or if it appeared in at least one of the significant interaction or square terms.

After term selection was performed, another response surface model was fitted using the selected terms. The resulting computed adjusted R^2 for the reduced model increased to 72.17% and the analysis of variance indicated that the linear, interaction, and square terms have significant contribution to the model. The regression model used was highly significant as indicated by the computed F-value of 40.84 compared to the critical F-value which is 1.85.

Moreover, a non-significant lack-of-fit (p-value=0.591) was obtained indicating that the model adequately described the experimental data. It was found out that the interaction of wort and CSL, wort and shaking or agitation, and CSL and yeast extract are significant, this means that their interactions affected the total yield. The new model had only 12 terms (including the intercept) as compared to the previous model which had 66 terms. Furthermore, the fit was improved as shown by the increase in adjusted R^2 value, so that the response is given by the following quadratic polynomial equations:

The model using uncoded units:

$$Y = 3.0965 + 0.03937^{*}\text{Whey} + 0.2252^{*}\text{Wort} - 0.1585^{*}\text{CSL} + 5.0626^{*}\text{PP} - 23.1169^{*}\text{YE} - 0.01089^{*}\text{Shaking} - 0.003416^{*}\text{Wort}^2 + 0.1063^{*}\text{CSL}^2 - 0.03652^{*}\text{Wort}^{*}\text{CSL} + 0.0005440^{*}\text{Wort}^{*}\text{Shaking} + 14.7291^{*}\text{CSL}^{*}\text{YE} \quad (2)$$

In Equation (2), Y is the response or lactic acid yield (g/L). It can be observed that whey, wort, CSL, and proteose peptone had positive contributions to the yield, whereas yeast extract had negative impact on the yield. The negative impact of yeast extract means that if the amount of yeast extract is increased, its main effect is to reduce the yield. Also, it seemed that the interaction of wort and CSL would decrease the amount of yield. The effect of the interaction of wort and CSL to the yield is best demonstrated using a contour plot, Figure 2a.

The graph showed clearly that if the effect of interaction to the lactic acid yield is to be reduced we have to increase the proportion of wort, and at the same time maintain or decrease the proportion of CSL.

Analysis of the interaction of wort and shaking revealed another evidence of the significant impact of wort to lactic acid yield. Figure 2b shows the contour plot of the effect of the interaction of wort and shaking to yield. Based from the contour plot, an increase in the amount of wort had a positive effect on lactic acid yield while shaking should be appropriately adjusted since it might have a negative impact on yield if the wrong proportions of the other components are used.

From Equation (2), an optimization to find the best combination of input to maximize the total lactic acid yield was done. The optimal point was found by doing a canonical analysis of the surface. The global

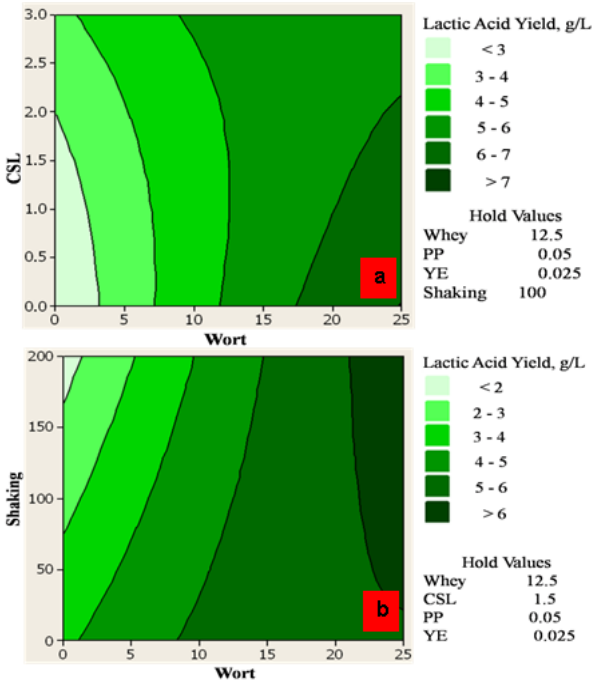

Figure 2. Contour plot showing the relationship between the interaction of CSL, wort, and lactic acid yield (a) and of shaking, wort, and lactic acid yield (b)

solution generated specifies the use of the following proportions and condition: whey (25%), wort (25%), CSL (0%), proteose peptone (0.1%), yeast extract (0%), and 200 rom shaking.

The desirability of these optimal levels is 1.0 which implies that the combinations of the levels of the input variables are within the bounds of the experiment. Additional confirmatory experiments were done around this optimal solution and near some local optimal solution to verify if the yield was actually better on these points (data not shown). After taking into account some practical considerations (survivability of bacteria, cost, energy input, etc.), and because *E. faecium* DMF78 is a facultative anaerobe, its growth will be sacrificed at high shaking speeds, hence making a compromise in the shaking speed and the proportions of the other components. As a consequence, the researchers made an executive decision to rather compare the performance of the microorganism using a 2-L fermentor than do validation in shake-flasks.

Fermentor run

Figure 3 shows the performance of *E. faecium* DMF78 in lactic acid fermentation using the optimized medium, with 1% sago starch, as monitored over a 48-hour fermentation in the2-L fermentor. The pH was kept at 6.5 with stirring at 48 rpm. Results showed that lactic acid level increased dramatically until the 32nd hour, reaching up to 35.32±1.252 g/L. Statistical analysis showed that lactic acid production at the 32nd, 40th and 48th hour were not significantly

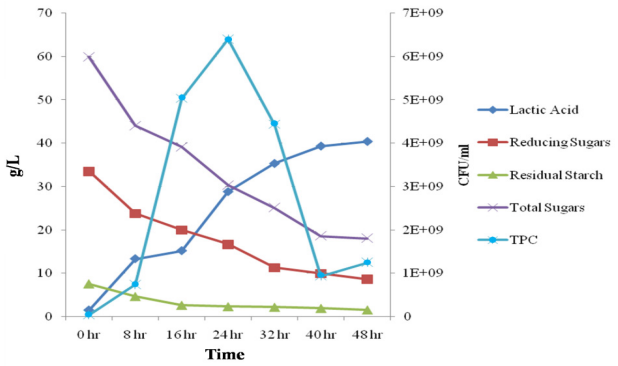

Figure 3. Time-course measurements of the direct lactic acid fermentation by *E. faecium* DMF78 using the new medium at pH 6.5

different. However, highest lactic acid productivity is attained after 24 hours of fermentation at 1.201 g/L-hr. The decrease in residual starch and reducing sugars were very minimal. Total plate count only reached 10^8 CFU/ml, whereas Shibata *et al.* (2007) had up to 10^{11} CFU/ml using starch-MRS medium. This showed a slow growth of the bacteria.

Any unusual increase in the lactic acid produced over the theoretical yield could be attributed to the fermentable sugars present in wort, whey and CSL, which could also be assimilated by the microorganism. From Figure 3, the total sugars is the sum of the reducing sugars and residual starch, whey lactose and maltose from wort (Briggs *et al.*, 1981; Ghaly *et al.*, 2003). Although it had a lower initial starch feed, the alternative medium had a higher maximum lactic acid content and yield (YLA/TS). Lower utilization of starch was also observed. This is due to the reducing sugars present, which acted in competition. Since they are easier to consume, they would be assimilated first. In general, in spite of the presence of other sugars, *E. faecium* DMF78 still hydrolyzed starch to lactic acid.

Conclusion

A response surface methodology was successfully carried out to optimize the production of lactic acid by *E. faecium* DMF78 using sago starch and low cost nitrogen sources such as whey, CSL and wort. Using the optimized medium described herein, an equivalent amount of lactic acid can be produced when using the standard formulation of MRS where glucose is substituted with sago starch. It should be especially emphasized that this medium has eliminated the need for yeast extract and uses only 0.78% of peptone in the original recipe of MRS.

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