Determination of EPS production

MRS media (20 ml) were inoculated (10%) with strains pregrown in MRS medium. After 3 days of incubation, 1 ml culture samples were centrifuged (4 min at 11000 g). Three volumes of cold (4 °C) ethanol were added to one volume of culture supernatants; the mixtures were stored overnight at 4 °C. Precipitates were collected by centrifugation (15 min at 2000 g) to remove cells using Sorvall™ RC 6 plus centrifuge (Thermo Fisher Scientific, Inc., USA), and resuspended in one volume of demineralized water. After precipitation with two volumes of cold ethanol and centrifugation, pellets were dried at 55 °C. EPS was determined by measuring the dry weight or total carbohydrate content of the precipitates. Total amount of EPS was determined by the total carbohydrate content of the precipitates by the phenol-sulfuric acid method using glucose as standard (Dubios et al., 1956). Briefly, 1 ml of EPS sample aliquots were mixed with 1 ml distilled water and 1 ml of 6% phenol aqueous solution and 5 ml of sulfuric acid 95% (v/v) were added quickly. After vigorously mixing, absorbance at 490 nm was measured using Multiskan™ GO microplate spectrophotometer (Thermo Fisher Scientific, Inc., USA). The concentration of EPS was determined in triplicate.

Experimental design by using Plackett-Burman design (PBD)

A PBD is used for screening multifactor to find the most significant independent factors. For screening purpose, various medium components were evaluated. The independent variables of the fermentation medium were Peptone, Lab lemco powder, dextrose, yeast extract, KH_2PO_4, $CH_3COONa.3H_2O$, $((NH_4)_3C_6H_5O_7)$, $MgSO_4 \cdot 7H_2O$, $MnSO_4.4H_2O$ and Tween 80. The 10 factors were investigated using the PBD with a first order polynomial equation. Each factor was conducted in 2 levels: -1 for low level and +1 for high level. Ten variables were screened in 12 experimental run. The data was carried out with the statistical package (State-Ease Inc., USA) and indicated that was significant factors ($p<0.05$). Placket-Burman experimental design is based on the first order polynomial model.

$$Y= \beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_4X_4+\ldots+\beta_{10}X_{10} \quad (1)$$

Y is the response, β_0 is constant, β_1-β_{10} are linear coefficients, X_1-X_{10} are the independent variables.

Effect of different carbon source

EPS production was screened in modified MRS media containing maltose (MRS-m), lactose (MRS-l), sucrose (MRS-s) and fructose (MRS-f) instead of the 20 g/l dextrose normally present in MRS medium (MRS-d). The initial pH was set at 6.8 by addition of 2 M or 4 M NaOH. All media were autoclaved at 121 °C for 15 min. In the preparation of modified MRS, the sugars were autoclaved separately. Infusion flasks (250 ml) equipped with a magnetic stirrer, incubated in an anaerobic glove cupboard, or Applikon (Schiedam, The Netherlands)/Bioflow III, flushed with nitrogen, were used for anaerobic batch fermentations at 37 °C. Each experiment described was performed at least in triplicate; data presented are averages with a standard deviation of less than10%.

Effect of initial concentration

The sugar concentration is another essential factor that influences EPS production. In this study, the effect of initial concentration was investigated at 2, 5, 10, 15, 20 and 25% w/v by adjustment with crystalline sugar. Fermentation was performed at 37°C, pH 6.8 in an anaerobic glove cupboard. EPS yield and cell growth was evaluated.

Effect of pH

pH is one of the most important factors which can influence growth and production of particular products by LAB. In this study, the EPS production was investigated in the

modified MRS-sucrose medium at pH 4.3, 5.5, 6.8, 7.0 and 7.5. Fermentation was performed at a temperature of 37°C in an anaerobic glove cupboard. EPS yield and cell growth was evaluated.

Response surface methodology for optimizing C-source concentration, pH and fermentation time

Central composite designs (CCD) and response surface analysis: Based on the results obtained in above experiments, the CCD was used to find the optimal concentrations of these significant factors, and to understand the relationship between the factors and EPS production. The carbon sources (sucrose) was chosen as the independent variables. EPS production (Y, g/L) was used as dependent output variables. For three variables (n =3) and five levels [low (-1.682) and high (+1.682)], 20 sets of experiments were employed for optimization of medium constituents. A 2^n CCD (2^3 = 8 factor points) plus 2n (2x3 = 6: axial points, with α = 1.682) and six replicates at the center points (n_0 = 6) was used. All experiments were carried out in triplicates. A multiple regression analysis of the data was carried out with the statistical package (Stat-Ease Inc., USA) and the second-order polynomial equation that defines predicted response (Y) in terms of the independent variables was obtained:

$$Y = \beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{23}X_2X_3 \quad (2)$$

Model validation and confirmation

The strain was preliminarily inoculated in The predicted condition which was obtained by statistical design was selected and confirmed by *L. fermentum* 484/24/3 by using MRS broth as basal medium at 37°C. The experiments were operated in screw vial and Duran bottle under anaerobic condition. Biomass and EPS were quantified. The percentage of derivation between the predicted and experimental value were investigated.

Results

Screening significant factors by PBD.

The primary components of fermentation medium, namely, Peptone, Lab lemco powder, dextrose, yeast extract, KH_2PO_4, $CH_3COONa.3H_2O$, $((NH_4)_3C_6H_5O_7)$, $MgSO_4{\cdot}7H_2O$, $MnSO_4.4H_2O$ and Tween 80 for EPS production were identified by PBD. The effects of these components on the response indicated that there was a wide variation of biomass from 0.21-1.73 g/l and EPS production from 0.89 to 1.83 g/L, respectively in the 12 trials. This variation reflected the importance of medium optimization to attain higher yields. Analyzed Design-Expert, a first-order model was fitted to the results obtained from the 12 experiments

$$Y_1\ (g/l) = 0.16+0.027X_1+6.481E^{-03}X_2+0.066X_3+1.235E^{-03}X_4\ -0.048X_5+0.016X_6-8.642E^{-03}X_7-0.18X_8-1.60X_9-0.11X_{10} \quad (3)$$

$$Y_2\ (g/l) = 0.81+0.016X_1+0.021X_2+0.036X_3+0.027X_4+0.12X_5-0.016X_6+0.14X_7-1.02X_8-3.00X_9-0.22X_{10} \quad (4)$$

where, Y_1 and Y_2 are the response for biomass and EPS production, respectively X_1, X_2, X_3, X_4, X_5, X_6, X_7, X_8, X_9 and X_{10} are the coded values of the test variables, Peptone, Lab lemco powder, dextrose, yeast extract, KH_2PO_4, $CH_3COONa.3H_2O$, $((NH_4)_3C_6H_5O_7)$, $MgSO_4{\cdot}7H_2O$, $MnSO_4.4H_2O$ and Tween 80, respectively. The regression equation obtained from analysis of variance (ANOVA) indicated that the correlation coefficient of R^2 is 0.9984 and 0.9963 which the model can explain 99.84% and 99.63% variation in the response for biomass and EPS, respectively. The t-test was used to identify the effect of every factor on EPS production. The data in Table 1 indicated that the dextrose was significant factors

($p<0.05$). Thus, the dextrose was selected for further optimization to obtain a maximum response.

Table 1 Variables investigation in the Plackett-Burman design

Factor	Source	*p>F*	
		DCW	EPS
Peptone	X1	0.1268	0.2182
Lab lemco powder	X2	0.5148	0.2077
dextrose	X3	**0.0263**	**0.0492**
yeast extract	X4	0.9423	0.3023
KH_2PO_4	X5	0.3270	0.1434
$CH_3COONa.3H_2O$	X6	0.3834	0.3911
$((NH_4)_3C_6H_5O_7)$	X7	0.8039	0.1273
$MgSO_4 \cdot 7H_2O$	X8	0.6290	0.1718
$MnSO_4.4H_2O$	X9	0.3788	0.2281
Tween 80	X10	0.2923	0.1615

Effect of different carbon source.

EPS production was screened in modified MRS media containing maltose (MRS-m), lactose (MRS-l), sucrose (MRS-s) and fructose (MRS-f) instead of the 20 g/l dextrose normally present in MRS medium (MRS-d). The initial pH was set at 7.0. After 48 h of cultivation, Biomass and EPS was determined. The result showed in figure 1. Growth in modified MRS media with the different sugar was fluctuated whereas dextrose, maltose and sucrose gave biomass insignificant and higher than lactose and fructose obviously. Biomass was about 1.98± 0.06 g/l. Final pH of culture medium was changed which trended toward to more acidity. EPS production was observed. Sucrose supported to EPS production as well that gave the highest value at 3.8±0.3 g/l so that sucrose was selected to further study.

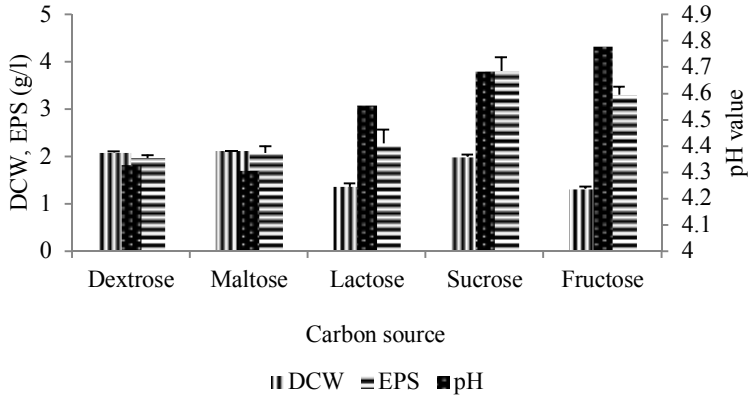

Figure 1: Growth, EPS production and pH change during cultivation of *L. fermentum 484/24/3* in MRS medium supplement with the different sugar as sole carbon at concentrations 20 g/l under anaerobic condition at 37ºC for 48 h

Effect of initial sucrose concentration.

The sugar concentration is another essential factor that influences EPS production. In this study, the effect of initial sucrose concentration was investigated at 2, 5, 10, 15, 20 and 25% w/v by adjustment with crystalline sucrose, pH 6.8 and incubated at 37°C. Biomass and EPS production was enhanced with the increase in initial sucrose concentration. Both biomass and

EPS production were increased continuously when higher sucrose concentration was occurred. 25% w/v sucrose was evident (Figure 2) which showed maximum biomass (7.59±0.09 g/l) and EPS (13.66±0.57g/L) after 48 h. pH was decreased in parabolic trend. It showed that sucrose was consumed and converted relatively into lactate.

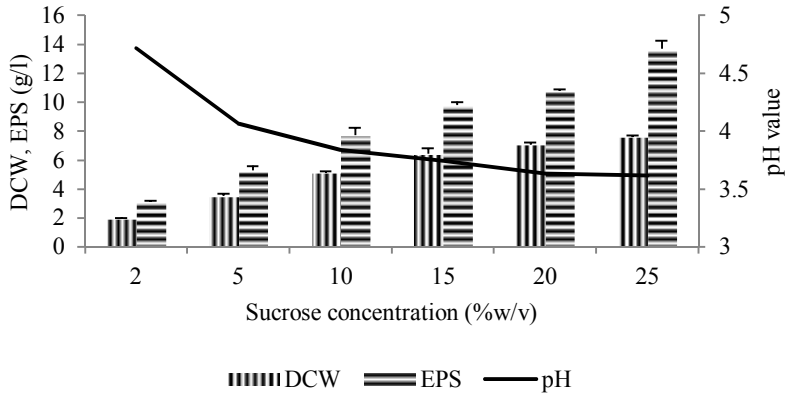

Figure 2: Growth, EPS production and pH change during cultivation of *L. fermentum* 484/24/3 in MRS medium (pH 6.8) supplement with sucrose as sole carbon at various concentrations under anaerobic condition at 37ºC for 48 h

Effect of pH.

pH is one of the most important factors which can influence growth and production of particular products by LAB. The EPS production was investigated in the modified MRS-sucrose medium with initial sucrose concentration of 25% w/v at pH 4.3, 5.5, 6.8, 7.0 and 7.5 incubated at 37°C. The favorable pH level for EPS production was found to be 6.8 because EPS which was produced in pH 6.8, 7.0 and 7.5 was insignificant. The highest EPS production was 14.3±0.26 g/L after 48 h incubation period (Figure 3). The maximum production level of lactic acid also occurred at this range of pH. Initial pH at neutral (6.5-7.5) was not affected to sucrose utilization whereas the biomass production was decreased obviously when initial pH was low.

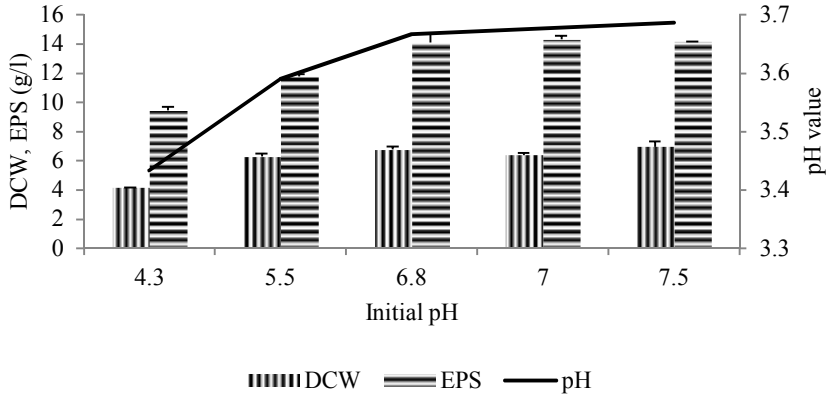

Figure 3: Growth, EPS production and pH change during cultivation of *L. fermentum* 484/24/3 in MRS medium supplement with sucrose as sole carbon at concentrations of 25% w/v by adjusted initial pH of medium in various value under anaerobic condition at 37ºC for 48 h

RSM for optimizing C-source concentration, pH and fermentation time.
Results of the 20 experiments for EPS production from *L. fermentum* 484/60/3 was observed while the second-order response surface models in the form of ANOVA were given in Table 2. Regression equation showed EPS was an empirical function of the test variables in coded unit. It was found to be significant ($P<0.05$) and lack of fit was not significant ($P>0.05$). Only significant terms were selected to receive the highest value of coefficient of determination (R^2) (Box et al., 1978) as shown in the Eqs. 5. In this study, the values of R^2 varied from 0.95 which suggested that the models gave good fit (Table 2) as shown in following equations:

$$EPS = -4.04+0.026x_1+2.75x_2-1.483E\text{-}03x_3+0.012x_1^2-0.074x_2^2-2.203E\text{-}04x_3^2 - 0.038x_1x_2+5.264E\text{-}03\ x_1x_3-4.236E\text{-}03x_2\ x_3 \quad (5)$$

where x_1, x_2 and x_3 represent codified levels of sucrose concentration, pH and fermentation time, respectively.The above equations was evaluated by the Fisher's F-test which showed very low probability value [($P_{model}> F)<0.0001$] whereas lack of fit value showed high probability value [($P_{model}>F)>0.05$]. This indicated that the model was highly significant (Table 2). The goodness of fit the model was determined by coefficient ($R^2 = 0.9565$ which implied that the sample variation of more than 95.65%) was attributed to the variables and only 4.35% of the total variance could not be explained by the model. The adjusted determination coefficient (Adj $R^2 = 0.9174$) was also satisfactory to confirm the significance of the model. A lower value of coefficient of variation (CV) indicated that the conducted experiments were precise and reliable (Box et al., 1978).

Table 2 ANOVA for full quadratic model of EPS production

Source	Sum of squares	Degree of freedom (DF)	Mean square	F-*value*	Prob > F
Model	113.18	9	12.58	48.95	< 0.0001
Residual	2.57	10	0.26		
Lack of Fit	0.96	5	0.19	0.60	0.7080
Pure Error	1.61	5	0.32		
Cor Total	115.75	19			
Root MSE	0.51				
Dep Mean	14.52				
C.V.	3.49				
$R^2 = 0.9778$	Adjust $R^2 = 0.9578$				

Response surface on EPS production was obtained. The result showed the studies on the effect of initial pH, sucrose concentration and fermentation time indicated the interaction between two parameters. Contour plots (2D) and 3D surface were generated to illustrate the interaction between two factors and optimum value of each factor affecting the response was achieved. Initial pH and sucrose concentration in figure 4 (a) showed that sucrose concentration was affected significant on EPS production whereas initial pH was had no effect at each pH. This result was expose similarly in the studies of the fermentation time and sucrose concentration in Figure 4(b) which gave higher EPS production when increased sucrose concentration whereas fermentation time was less affected when cultures for longer time. Finally, the interaction between fermentation time and initial pH was evident that interaction was negligible between the corresponding variables. The insignificance of EPS production was occurred shown in figure 4(c) although fermentation time or initial pH had adjusted but EPS production was quite constant. Verification of the model in screw vial and duran bottle under anaerobic condition was achieved. Both conditions gave the highest biomass and EPS at 48 h about 7.74±0.46 g/l and 17.21±0.30 g/l, respectively. The selected condition was conducted by using (25, 6.7, 39.48) and (25, 6.2, 43.89) for initial

concentration, pH and fermentation time, respectively. The result gave that predicted value and experimental value (g/l) was not significant which suggested that the models gave good fit.

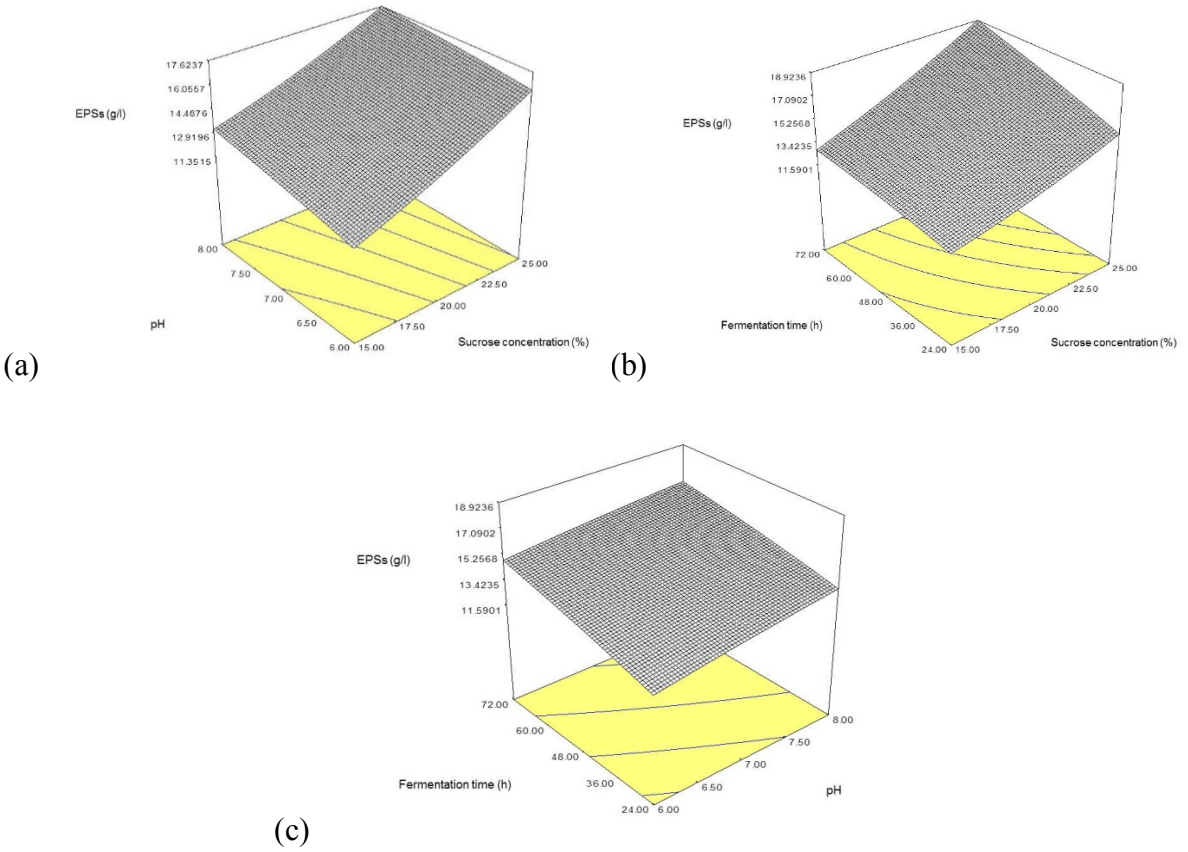

Figure 4: RSM on EPS production (a) between pH and sucrose concentration (b) between fermentation time and sucrose concentration (c) between fermentation time and pH

Discussion

The total yield of EPS produced by LAB depends on the composition of the medium and the environmental conditions in which microorganism grow such as carbon source, concentration, initial pH and fermentation time (De Vuyst and Degeest, 2001). Plackett-Burman design was used to screen the reliable factor (Bereck et al., 2010). Sucrose was the most efficient carbon source or precursor for EPS production whereas lactose and fructose were not an efficient carbon source. For instance, increasing sucrose concentration resulted in concentration of EPS obviously. The concentration dependent increase in EPS production was correlated with growth. Initial pH of culture medium was shown that was not affected significantly in range of 6.5-7.5 which often found in LAB (Zhang et al., 2011) whereas low pH was prohibited growth because enzymes mechanism was exhibited to slower cell wall polymer synthesis and isoprenoid phosphate had no enough for EPS synthesis (Cerning et al., 1994). Growth and EPS production were correlated by fermentation time because EPS was probably secreted along growth process so that EPS was occurred in high concentration after last period of exponential phase of growth. RSM was conducted to observe interaction between factors which exposed that sucrose concentration enhanced EPS production although pH and fermentation time was adjusted.

Conclusion

EPS production in modified MRS to study the influence of different conditions on the biopolymer and the cell growth. Single factor experiments were used to determine the optimum range of several parameters and then response surface methodology with Central composite design was subsequently applied to determine the effects of significant parameters and their interactions and to identify the optimum values. Finally, the optimum conditions were experimentally validated. The study showed that EPS production by *L. fermentum* 484/24/3 could be improved by optimization of medium and environmental conditions such as carbon source, initial carbon concentration, initial pH including fermentation time. Optimization was studied by using statistical methodology both Plackett-Burman and response surface methodology. Comparison of growth and EPS production between original condition and statistical optimized condition was obtained which increased EPS about 9.25 fold (from 1.9±0.17 to 17.57±0.61 g/l) whereas 3.3 fold for biomass from 2.36±0.04 to 7.8±0.34 g/l.

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