CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Bacterial strain

EPS-producing *P. urinae-equi* TISTR 1499 which was isolated from Thai fermented food "Nham" (Wongroung *et al.*, 2002) and identified by Maungtoom *et al.* (2002) was employed. This strain was subcultured monthly in 5 mL Man, Rogosa and Sharpe (MRS) agar slant, incubated at 37°C for 12 h and stored at 4°C.

3.1.2 Culture medium

Man, Rogosa and Sharpe (MRS) medium (modified from Degeest and de Vuyst, 2000) was used as the basic EPS production medium; one liter of it contains 10 g peptone, 5 g yeast extract, 8 g meat extract, 2 g K_2PHO_4, 5 g sodium acetate, 2 g diammonium hydrogen citrate,

0.1 g $MgSO_4.7H_2O$, 0.05 g $MnSO_4.H_2O$, 1 mL tween 80 and 20 g sucrose . The same medium composition was used for cultivation on agar plate by addition of 2 % agar. The medium was autoclaved at 110°C for 25 min.

3.1.3 Solid support materials

(1) Agar support culture

MRS agar medium was performed using 100 mm diameter Petri dishe filled with 20 mL of medium or desired volume.

(2) Synthesized solid support materials

(2.1) Paper put on cellulose sponge, which was prepared by cutting 80 gram paper (Tesco-Lotus, Thailand) and cellulose sponge (3M, Thailand) into 4 x 6 cm.

(2.2) Terra-cotta (Sriladol, Thailand), which was cut into 4 x 6 cm.

(2.3) A plastic bead (Chiang Mai Plastic, Thailand), which has a diameter of 5 mm.

(2.4) Polystyrene sponge (3M, Thailand), which was cut to size 5x5x5 mm^3

(3) Agricultural waste support materials

(3.1) Rice husk, which was collected from a rice mill in Mae-Hea district, Chiang Mai province, Thailand.

(3.2) Rice straw, which was collected from paddy field in Mae-Hea district, Chiang Mai province, Thailand.

(3.3) Sugarcane bagasse, which was collected from sugar factory in Lampang province, Thailand.

3.1.4 Media and chemical reagents

Name of chemical reagents	**Production company**
Bacto-Peptone	HIMEDIA
Beef extract	HIMEDIA
Yeast extract	HIMEDIA
Sucrose	Mitrpol
Tween 80	LABCHEM

Name of chemical reagents	Production company
K_2HPO_4	MERCK
KH_2PO_4	MERCK
$(NH_4)SO_4$	Scharlau
Di-ammonium hydrogen citrate	UNILAB
$MgSO_4.12H_2O$	UNILAB
$MnSO_4$	Scharlau
Conc.HCl	MERCK
95 % Ethanol	OV chemical

3.1.5 Equipments

Name of equipments	Production company
Analytical balance (4 decimal)	OERTING
Autoclave Model ACV-3167	IWAKA

Name of equipments	Production company
Hot-air oven	MEMMERT
Laminar air flow cabinet	LABCONCO
Hood	TOPLAB
Autopipette	GIBTHAI
Refrigerated centrifuge (Model Super T21)	SORVALL
Centrifuge (Model Harmonic series)	GEMMY
pH meter (Model C830)	CONSORT
Spectronic 21	GENESYS
Microscope	OLYMPUS
Microwave Model R-242	SHARP
Vortex Mixer (Model 2 Genie)	BOHEMIA
Water bath Model 1255 PC	SHEL-LAB

Name of equipments	Production company
Air pump	STY
Fermentor (5 L)	BIOSTAT

3.2 Methods

3.2.1 Inoculum preparation

Sterilized Erlenmeyer flask containing 50 mL of MRS medium was inoculated with a full loop of a freshly *P. urinae-equi* TISTR 1499 culture. After incubation at 37°C for 12 h on rotary shaker, this pre-culture was used as inoculum.

3.2.2 Fermentation procedure

(1) Submerged culture for EPS production

Fermentation was carried out in 1 L Erlenmeyer flask containing 450 mL of the culture medium. After sterilization, it was inoculated with 10% (v/v) (50 mL) of *P. urinae-equi* TISTR 1499

culture. The inoculated flask was incubated on a rotary shaker at 37°C and 200 rpm for desired time intervals.

(2) Agar support culture for EPS production

Cultivation on agar medium surface was performed using 100 mm diameter Petri dishe filled with 20 mL of MRS agar medium. The agar medium was inoculated by spreading of the 0.2 mL inoculum. The cultivation was incubated at 37°C in the chamber system suppling with moist-air by pumping fresh air pass through steriled water (Fig. 3.1).

(3) Synthesized solid support culture

The cultivation on Terra-cotta and paper put on cellulose sponge was prepared by immersing a sterilize solid support in MRS liquid medium and then incubated at 37°C for 24 h. After that, the solid support was transferred into the 100 mm diameter Petri dish with aseptic technique. The surface of Terra-cotta and paper were inoculated by spreading of 0.2 mL inoculum. The cultivation was incubated at 37°C in the chamber system suppling with moist-air.

The cultivations on plastic beads and polystyrene sponge were performed by using 250 mL Erlenmeyer flasks filled with plastic beads or polystyrene sponge and MRS medium in the ratio of 1 : 3 (w/v) and 0.1 : 3 (w/v), respectively. After immersing with the liquid medium, the mixture was autoclaved at 121°C for 20 min. Then, the mixture was inoculated with 10% (v/v) of inoculum and cultivated at 37°C on a rotary shaker at 200 rpm for desired time intervals.

(4) Agriculture waste support culture

The agricultural wastes (rice husk, rice straw and sugarcane bagasse) were individually pretreated by boiling in 2 M HCl for 20 minutes. Then, the treated materials were neutralized to pH 7.0 with 2 N NaOH and dried in a hot-air oven at 50°C overnight. After that, 7 g of each treated materials was added into 250 mL Erlenmeyer flasks and mixed with MRS liquid medium in the ratio of 1 : 3 (w/v). The mixtures were autoclaved at 121°C for 20 min. After inoculation with *P. urinae-equi* TISTR1499, the mixtures were incubated at 37°C at 200 rpm for desired time intervals.

3.2.3 Optimization of fermentation process parameters for EPS production under solid support culture

3.2.3.1 Optimization of fermentation process parameters on agar plate culture

(1) Effect of moist-air supplement on EPS production

The fermentation system with moist-air was operated in 2 L glass covered chamber with 2 ports for moist-air inlet and outlet (Fig. 3.1). The moist-air was produced by pumping sterilized air through sterilized water and passed into the chamber. The system except the pump was autoclaved at 121°C for 20 min. The fermentation system was kept in plastic box with cover during cultivation (Fig. 3.2).

Fig. 3.1 The fermentation system with moist-air

Fig. 3.2 The fermentation system kept in the plastic box

(2) Effect of moist-air flowrate, incubation time and MRS agar medium on EPS production

To determine the effect of some parameters affecting EPS production by *P. urinae-equi* TISTR 1499, a response surface experimental design using a quadratic model was employed by using the DESIGN-EXPERT Software, Version 5.0 (Stat-Ease, Inc., Minneapolis, MN). A central composite design (CCD) with three variables was used to study the response pattern and to determine the optimum combination of variables. The variable optimizations were moist-air flowrate (7 to 28 mL/min), incubation time (17 to 31 h) and MRS agar medium volume (10 to 18 mL) which were studied each at 5 levels, -1.682; -1; 0; 1; 1.682 (Table 3.1).

Table 3.1 Variables and their levels for CCD

Variables	Level				
	-1.682	-1	0	1	1.682
Moist-air circulation (mL/min)	0	7	17	28	35
Incubation time (h)	12	17	24	31	35
MRS medium volume (mL)	7	10	14	18	20

The CCD is shown in Table 3.2. The table was arranged to allow for fitting an appropriate regression model using multiple regression programs. The CCD combines the vertices of the hypercubes whose co-ordinates are given by a 2^n factorial design to provide for the estimation of curvature of the model. Experiments were randomized in order to maximize the effects of unexplained variability in the observed responses due to extraneous factors.

(3) Effect of initial sucrose concentration on EPS production

Different concentrations of initial sucrose (3, 6, 9, 12, 15, 18 and 20 g/L) in MRS agar medium were studied. The other conditions for cultivation were kept at the optimum conditions.

3.2.3.2 Optimization of fermentation process parameters using rice husk as solid support

(1) Effect of the ratio of rice husk to MRS medium on EPS production

Different ratios of rice husk to MRS medium (1:1, 1:2, 1:3, 1:4 and 1:5 w/v) were determined. After sterilization and inoculation, the inoculated flasks were cultivated on a rotary shaker at 37°C and 200 rpm for desired time intervals.

Table 3.2 Treatment schedule for a three-factor CCD

Std	Run	Moist-air flowrate (mL/min)	Incubation time (h)	MRS medium volume (mL)
4	1	7	31	18
5	2	28	17	10
1	3	7	17	10
12	4	17	24	14
6	5	28	17	18
10	6	17	24	14
3	7	7	31	10
8	8	28	31	18
2	9	7	17	18
9	10	17	24	14
11	11	17	24	14
7	12	28	31	10
17	13	0	24	14
14	14	17	24	20
18	15	35	24	14
19	16	17	24	14
16	17	17	35	14
13	18	17	24	7
15	19	17	12	14
20	20	17	24	14

(2) Effect of inoculum size on EPS production

Different percentages of inoculum size (2.5, 7.5, 10, 12.5, 15, 20 and 25 %) were studied. The ratio of rice husk to MRS medium was employed at 1-to-3 (w/v). The inoculated flasks were cultivated on a rotary shaker at 37 °C and 200 rpm for desired time intervals.

(3) Effect of initial sucrose concentration on EPS production

Different concentrations of initial sucrose (10, 15, 20, 25, 30, 40, 50, 60, 70 and 80 g/L) were investigated in Erlenmeyer flasks. The ratio of rice husk to MRS medium was employed at 1-to-3 (w/v). After sterilization, the flasks were inoculated with 15 % inoculum. The inoculated flasks were cultivated on a rotary shaker at 37 °C and 200 rpm for desired time intervals.

(4) Effect of nitrogen sources on EPS production

To determine the effect of various nitrogen sources on EPS production by *P. urinae-equi* TISTR 1499, a response surface experimental design using a quadratic model was employed by using the DESIGN-EXPERT Software, Version 5.0 (Stat-Ease, Inc., Minneapolis, MN). A mixture design with four variables was used to study the response pattern and to determine the optimum

combination of variables. The variables optimized were concentration of yeast extract (0 - 1%), meat extract (0 - 1%), bacto-peptone (0 – 1.5%) and di-ammonium hydrogen citrate (0 –0.5%) (Table 3.3). Each experiment was controlled the total nitrogen source of 1.50% (w/v).

Table 3.3 Treatment schedule for a four-factor mixture design using rice husk as solid support

Std	Run	Yeast extract (%)*	Meat extract (%)*	Bacto-peptone (%)*	Di-ammonium hydrogen citrate (%)*
16	1	0.00	0.00	1.50	0.00
4	2	1.00	0.00	0.00	0.50
18	3	0.00	1.00	0.00	0.50
13	4	0.50	0.50	0.50	0.00
20	5	0.25	1.00	0.00	0.25
7	6	0.00	0.00	1.00	0.50
17	7	0.00	1.00	0.50	0.00
2	8	1.00	0.50	0.00	0.00
10	9	0.50	0.50	0.00	0.50
15	10	0.33	0.33	0.33	0.50
6	11	1.00	0.00	0.50	0.00
19	12	1.00	0.50	0.00	0.00
14	13	0.72	0.22	0.22	0.34
3	14	0.00	1.00	0.00	0.50
5	15	0.00	1.00	0.50	0.00
12	16	0.50	0.00	1.00	0.00
9	17	0.25	1.00	0.00	0.25
8	18	0.00	0.50	0.75	0.25
11	19	0.50	0.00	0.50	0.50
1	20	0.00	0.00	1.50	0.00

* (%) defined by DESIGN-EXPERT version 5.0 program

In a mixture the sum of the components always add up to 100%. This is the mixture constraint. It will explain how mixtures can be optimized with the sequential simplex method, not to be confused with the simplex-lattice or simplex-centroid mixture designs.

The sequential simplex method can easily handle the mixture constraint and the dependence between the control variables. The work procedure is straightforward. The first simplex is defined in a mixture component space with one of the components left out. In multisimplex the classical first simplex may be easier to fit within the mixture boundaries, than the standard modified design.

Another way to carry out the mixture experiments is to keep all components as control variables, but forcing the simplex to move within a constrained mixture component space. In all trials the sum of the components must add up to 100%. One way of doing this is to specify the extreme vertices and the center point as the first simplex trials.

(5) Effect of moist-air supplement on EPS production

The fermentation system with moist-air was operated as described in 3.2.3.1 (1) and showed in Fig 3.3 and 3.4.

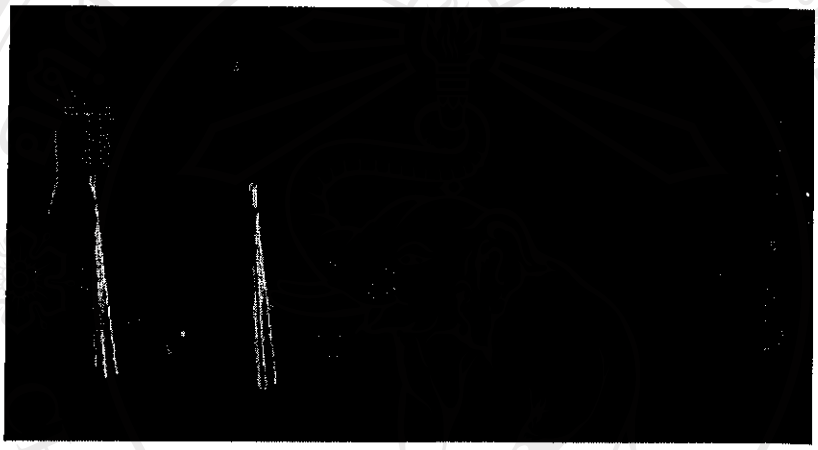

Fig. 3.3 The fermentation system for EPS production using rice husk as solid support with moist-air supplement

Fig. 3.4 The fermentation system for EPS production using rice husk as solid support with moist-air supplement in the plastic box

3.2.4 Recovery and quantity of cell dry weight and native EPS

3.2.4.1 Products obtained from submerged culture

At required time intervals, 20 mL of fermentation broth was withdrawn aseptically from the respective flask and centrifuged at 10,000*xg* for 20 min at 4°C to separate cell mass and supernatant. The cell pellet was washed one time with 20 mL deionized water and centrifuged at 10,000 *xg* for 10 min at 4°C. After that, the cell pellet was collected and dried at 60°C in a hot-air oven for 24 h to obtain a constant weight. Then, the cell dry weight was determined and expressed in g/L. A measured volume of the cell free supernatant (20 mL) was added into two volumes of cold 95 % ethanol (40 mL) and the ethanol-supernatant mixture was kept overnight at 4°C to precipitate EPS. The precipitate formed was then recovered by centrifuging at 10,000*xg* for 20 min at 4°C. The EPS pellet was washed one time by 95% ethanol 20 mL and centrifuged at 10,000*xg* for 10 min at 4°C. The precipitated EPS was then dried at 60°C for 24 h to obtain a constant weight. The dried EPS was weighed and expressed in g/L.

3.2.4.2 Products obtained from agar culture, terra-cotta culture and paper put on cellulose sponge culture

At required time intervals, the slimy growth on the surface of solid support was scraped off with a spatula and collected in a 50 mL centrifuge tube. The slime was suspended with 10 mL of distilled water and centrifuged at 10,000*xg* for 20 min at 4°C to separate the cell mass and supernatant. The cell pellet and cell-free supernatant were determined by the procedures as described in 3.2.4.1.

3.2.4.3 Products obtained from plastic beads culture, polystyrene sponge culture and agricultural waste culture

At required time intervals, the EPS and cell mass were extracted from the fermented mass by adding 100 mL of deionized water and shaked vigorously for 10 min. Solid support particles were removed by filtration through a fine nylon textile. The filtered mixture was centrifuged at 10,000*xg* for 20 min at 4°C to separated cell mass and supernatant. The cell pellet and cell-free supernatant were determined by the procedures as described in 3.2.4.1.

3.2.5 Analytical methods

3.2.5.1 EPS yield was determined by weighing of the dried EPS pellets (g/L MRS medium)

3.2.5.2 Cell growth was determined by determining the cell dry weight (g/L MRS medium) after drying at 60°C for 24 h.

3.2.5.3 Residual sugar remaining in culture broth was determined by using DNS method (Miller, 1959). One mL of sample, which removed cell and native EPS, was hydrolyzed with 4 mL of 2M HCl at 100°C for 15 min (George and Ghose, 1974) and followed by assaying with DNS method (Miller, 1959).

3.2.5.4 Kinetic parameters were determined for Maximum specific growth rate (μ_{max}), specific rate product formation (q_p), specific rate substrate consumption (q_s), the yield coefficient of biomass from substrate ($Y_{x/s}$), the yield coefficient of product from substrate ($Y_{p/s}$) and the yield coefficient of product from biomass ($Y_{p/x}$).

The kinetic parameters were calculated by;

μ_{max} (h^{-1})was calculated from Lineweaver-Burk plot by the $1/\mu_{max}$ is the Y axis intercept point value (Nagai, 1979).

$$Y_{X/S} = \Delta X / \Delta S \text{ (g cell dry weight / g substrate)}$$

$$Y_{P/S} = \Delta P / \Delta S \text{ (g EPS dry weight / g substrate)}$$

$$Y_{P/X} = \Delta P / \Delta X \text{ (g EPS dry weight / g cell dry weight)}$$

$$q_P = \mu * Y_{P/X} \text{ (g EPS dry weight / g cell dry weight / h)}$$

$$q_S = \mu / Y_{X/S} \text{ (g substrate / g cell dry weight / h)}$$

Where ΔX, ΔP and ΔS are the differential values of cell dry weight, EPS dry weight and sucrose concentration from initial to end of log phase, respectively (Nagai, 1979).

The μ is $(\Delta X / \Delta T) * 1/X$. Where ΔT is the differential values time from initial to end of log phase and X is the central value of cell dry weight between initial and end of log phase (Nagai, 1979).

3.2.6 Statistic analysis

All experiments were carried out in three replications and the mean values ± S.D. are reported. The results were submitted to respond surface analysis and ANOVA analysis by using the DESIGN-EXPERT Software, Version 5.0 (Stat-Ease, Inc., Minneapolis, MN). The statistical analysis had a significant lack of fit ($P < 0.05$) with the response surface model.

The Tukey's test (HSD) was used as the significant different test for compared the mean value of each treatment in each affect factor and significantly different at $P < 0.05$ for determination of the differentiation.