CHAPTER III

MATERIALS AND EXPERIMENTAL METHODS

3.1 Materials

3.1.1 Microorganisms

E. fibuligera 5097 was obtained from Thai Institute of Science Technological Research, Bangkok, Thailand (TISTR). They were maintained on YM agar slants. All cutures were stored at 4°C and were transferred every four week. E. fibuligera 5097 isolated from a mould bran in Thailand by Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok, Thailand (E. fibuligera DMKUY-1285). This strain produced two extracellular amylase, glucoamylase and an endolytic type of amylase -hydrolyzing cyclodextrins. It showed maximal activity at pH 5.5 and 60°C.

C. utilis 5001 was obtained from C.P. Kurtzman of the Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Illinois, U.S.A. (C. utilis NRRL Y-900).

3.1.2 Chemicals

Chemicals used in the experiment are as follows:

- $(NH_4)_2HPO_4$ Di-ammonium sulphate
- $(NH_4)_2SO_4$ Ammonium sulphate
- KH_2PO_4 Mono-potassium phosphate
- $MgSO_4$ Magnesium sulphate
- NaOH Sodiumhydroxide
- Na_2CO_3 Sodium carbonate

- Na_2SO_4 Sodium sulphate anhydrous
- $CuSO_4$ Cupric sulphate granule
- $(NH_4)_6Mo_7O_{24}.H_2O$ Ammonium heptamolybdate
- $K_2S_2O_5$ Potassium-metabisulfite
- Folin reagent
- Glucose
- Peptone
- Malt extract
- Yeast extract
- Agar
- Bovine Serum Albumin
- Antifoam: adecanol, vegetable oil

3.1.3 Media

The media given below are used for propagating of agar slants :

YM AGAR: glucose 10 g, peptone 5 g, malt extract 3 g, yeast extract 3 g, agar 20 g and distilled water 1 litre

YEAST-STARCH AGAR: yeast extract 2 g, soluble starch10 g, agar15 g distilled water 1 litre and adjust pH to 7.3

3.1.4 Starch

Cassava starch from cassava chips was used for the fermentation substrate. It contains 68 % starch, 18 % moisture, 3 % sand, 11 % raw fiber and other materials. Cassava chips were milled into powder and stored in a refrigerator at 4 ^{o}C. In broth preparation, the casava powder was mixed with some water (about 20%) and then boiling water (the remaining 70%) was added to gelatinize the starch. The gelatinized starch is chemically reactive for conversion with enzymes. The insoluble cellulose residue was removed afterward by filtration. The concentration of the cassava in the broth was

controlled by the quantity of water added.

3.1.5 Molasses

Molasses is the by-product from the saccharose extraction process from sugar-cane. Molasses using for the experiment was taken from Sura Saengsom company, Nakornpathom province, Thailand. The glucose content was determined to be 21 %. The total sugar is reported by the factory to be 39 % for this lot. Molasses used was stored in a refrigerator as well as the cassava.

3.2 Apparatus

3.2.1 Reactor set

The reactor used in this research consists of a 60-litre jacketed vessel, mechanically controlled variable speed motor, a set of impellers, air-sparger, controlled temparature water bath and a pH analyzer.

The fermentation vessel is an all-welded, jacketed tank with a removable head. The vessel is fabricated from 304 stainless steel. The cylindrical vessel inside diameter is 42 cm. The vessel has two removable stainless steel baffles. The width of each baffle is 3 cm. Baffle height is adjustable by certain fixtures on the edge the baffle and the innerwall of the vessel.

The impellers are a shrouded flat-blade turbine and a flat-blade paddle type. The turbine is located closed to the vessel bottom, it has six blades. The two-blade paddle is placed at 11.4 cm above the first impeller. The diameter of the two impellers are 1/2 that of the vessel. The shaft can be rotated with a variable speed motor adjustable from 30 to 200 rpm.

The air delivery system includes valves, a pressure regulator, a rotameter, and an air filter. The rotameter is capable to measure air flow rate over a range from 0.2 to 2.0 volme of air per volume of liquid per minute (vvm). Air is supplied jrom an air compressor.

The temperature inside the vessel is continually controlled by circulating water from water bath through a jacket around the vessel. The accuracy of temperature control is $\pm$ 1°C.

3.2.2 Other equipment

The pH of reactor content can be measured using a pH Analyzer model 1054 A and pH/ORP sensor model 399 from resemount Analytical Inc., USA

The concentration of reducing sugar and protein is determined by spectrophotometric method using a Spectronic 21 from BAUSCH & LOMB, cat No. 33.22.42., series No.100 1775, made in USA

The biomass is concentrated using a centrifuge model KC-25, KUBOTA from Japan.

Sterillisation of media is carried out using an autoclave model HA-30, Hirayama Manufacturing Co., Japan.

It is often required to heat cassava paste and other materials. this is conveniently achieved using a microwave oven model TRX-2500 BS, Turbo international Co.,Ltd., Japan.

Experiment with monoculture and preparation of starter for the 60-litre tank can be carried out using a large rotary shaker made by the workshop of the Department of Chemical Technology, Thailand.

3.3 Experimental methods

3.3.1 Experiment on the growth of Candida utilis 5001 in a monoculture.

C. utilis 5001 was grown in 17 flasks of 250ml with YM medium. Flasks were autoclaved for 20 minutes at 121°C and set on a rotary shaker with agitation speed of 150 rpm at 30°C for 48 hours. For every 3 hour one flask was taken for analysis of dried biomass, protein content, and glucose. It was found that the biomass, protein contents are relatively unchanged after 24 hours of cultivation. The kinetic parameters such as specific maximum growth rate (μ_{max}), specific substrate consumption rate (q_s), product yield of biomass ($Y_{p/s}$) were calculated. The method of calculation is in Appendix 3. In light of the above experiment, threerepeated series of experiment each with 5 observations were perfomed to determine the average dry weight after 24 h of cultivation.

3.3.2 Experiment on the growth of Endomycopsis fibuligera 5097 in a monoculture.

The analogous experiments were perfomed with E. fibuligera 5097 using yeast starch medium. Dried biomas, protein content, glucose and starch were determined. After 39 hours of cultivation, the results showed little change. The kinetic parameters such as specific maximum growth rate (μ_{max}), specific substrate consumption rate (q_s), product yield of biomass ($Y_{p/s}$) were calculated. The method of calculation is in Appendix 3. Three series of experiment each with 5 observations were therefore perfomed to determine the average dry weight at 39 hours of cultivation.

3.3.3 Experiment on the effect of introduction of C. utilis into the cultivating E. fibuligera

used for the cultivation and autoclaved for 20 minutes at 121°C. Flasks were shaken on rotary shaker at 150 rpm and 30°C. The seed culture was in the beginning E. fibuligera and later at a preset time C. utilis was added at a volume ratio to the E. fibuligera seed of 1:1, 1:2, 1:3 and 1:4. The preset introduction times of C. utilis into the cultivation were the 0th, 6th, 12th, 18th and 24th hour after inoculated by E. fibuligera. This experiment was repeated three times. Collection of samples were made after 48 hours of cultivation. Dry weight of biomass was determined by centrifugation of the cell suspension, the sediment was washed and then dried for 16 hours at 105°C.

3.3.4 Experiment on the use of molasses as a vitamin source for the production of SCP

Mixed culture of E. fibuligera and C. utilis was grown in 250 ml flasks. Molasses was used with ratios of 1:4, 2:4, 3:4, 4:4 to cassava in the medium. The cassava concentration were prepared at four lavels of 10 g, 15 g, 20 g, 25 g per litre. Threfore, there were a total of 16 formulas of medium for this expriment. These formulas were coded as M1 to M16. Detail of composition of medium are presented in table 3.1, 3.2

Table 3.1 Medium composition of M1 - M8

Medium	M1 g/l	M2 g/l	M3 g/l	M3 g/l	M5 g/l	M6 g/l	M7 g/l	M8 g/l
Cassava	10	10	10	10	15	15	15	15
Molasses	2.5	5.0	7.5	1.0	3.75	7.5	11.3	15
$(NH_4)_2HPO_4$	1.83	1.99	2.13	2.33	2.74	3.00	3.25	3.50
$(NH_4)_2SO_4$	0.91	0.99	1.06	1.16	1.37	1.50	1.62	1.75
KH_2PO_4	0.46	0.50	0.53	0.59	0.69	0.75	0.81	0.88
$MgSO_4$	0.4	0.05	0.05	0.06	0.07	0.08	0.08	0.09
pH	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5

The pH was kept constant at 5.5 with sodium hydroxide. All cultivating flasks were placed on a rotary shaker at 140 rpm and 30°C for 48 hours. For each formula, a sample was taken at 3 hours interval for analysis.

Table 3.2 Medium composition of M9 - M16

Medium	M9 g/l	M10 g/l	M11 g/l	M12 g/l	M13 g/l	M14 g/l	M15 g/l	M16 g/l
Cassava	20	20	20	20	25	25	25	25
Molasses	5	10	15	20	6.25	12.25	18.75	25
$(NH_4)_2HPO_4$	3.65	4.00	4.33	4.66	4.48	5.00	5.42	5.83
$(NH_4)_2SO_4$	1.82	1.90	2.16	2.33	2.24	2.50	2.71	2.91
KH_2PO_4	0.92	1.00	1.08	1.17	1.13	1.25	1.35	1.46
$MgSO_4$	0.09	0.10	0.11	0.12	0.11	0.13	0.14	0.15
pH	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5

3.3.5 Scale-up of SCP production on 60-litre tank

A mixed culture of E. fibuligera and C. utilis was grown in a 60-litre fermentor. The suitble medium was selected from the experiment described in section 3.3.4. However, $K_2S_2O_5$ was added at 100 ppm to prevent possibility of contamination. Cassava starch was prepared as described previously. The pH was kept constant at 5.5 with sodium hydroxide and temperature was maintained at 30°C. The seed culture was 8%. The agitation speed was 140 rpm and aeration rate 8 litre per minute, using medium M1, M16, M17, M18, M19 shown in Table 3.3.

Table 3.3 Medium composition of M17 - M19

Medium	M17 g/l	M18 g/l	M19 g/l
Cassava	35	35	35
Molaases	17.5	26.75	8.75
$(NH_4)_2HPO_4$	7.00	7.58	6.39
$(NH_4)_2SO_4$	3.40	3.78	3.19
KH_2PO_4	1.75	1.89	1.61
$MgSO_4$	0.18	0.19	0.16
pH	5.5	5.5	5.5

3.4 Analysis of subtrate and products.

Starch was determined as the difference between the total carbonhydrate and reducing sugars concentration. Total carbonhydrate and reducing sugars were determined by Somogyi-Nelson method as glucose equivalents as described in details in Appendix 1. The concentration of glucose is also determined by Somogyi-Nelson method

The biomass were removed by centrifugation and dried weight were analysed by gravimetric method.

The protein concentration of biomass is determined by Lowry method described also in Appendix 1.