CHAPTER 3
MATERIALS AND METHODS

3.1 Raw materials

3.1.1 Three years old dried longan flesh (obtained from Mrs. Sopit Boonkhum, 53, Moo 7, Makham-Luang sub - district, Sanpatong district, Chiang Mai province).

3.2 Microorganisms

3.2.1 *Saccharomyses cerevisiae* TISTR 5606 (Thailand Institute of Scientific and Technology Research, TISTR).

3.2.2 *Candida utilis* UNSW 709400 & 709700 (The University of New South Wales, UNSW).

All of the microbial strains were prepared in the form of working culture stock kept in 16% (v/v) glycerol solution. Each stock was kept in the 1.0 ml aliquot of 1.5 ml eppendorf tube and stored at -20°C before used.

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3.3 Chemicals and enzymes

Table 3.1 The list of chemicals and enzymes used

Names	Formula	Suppliers
Acetaldehyde	C_2H_4O	Riedel, GmbH, Germany, Cat. No. 60471
Acetic acid, glacial	CH_3COOH	Labscan, Dublin, Ireland, Cat. No. A8401
Acetonitrite	CH_3CN	Merck, Darmstadt, Germany, Cat. No. 1. 14291. 4000
Alcohol dehydrogenase (ADH)	-	Sigma, Steinheim, Germany, Cat. No. A7011
Ammonium sulphate	$(NH_4)_2SO_4$	Merck, Darmstadt, Germany, Product No. 168355
Benzaldehyde	C_6H_5CHO	Poch, Sowinskiego, Poland, Cat. No. 161530427
Coloured coded buffer pH 4	-	Ajax FineChem, Wellington, New Zealand, Cat. No. A2490
Coloured coded buffer pH 7	-	Ajax FineChem, Wellington, New Zealand, Cat. No. A2491
Coloured coded buffer pH 10	-	Ajax FineChem, Wellington, New Zealand, Cat. No. A2564
Ethanol, absolute	C_2H_5OH	Merck, Darmstadt, Germany, Product No. 1. 00983. 2500

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Table 3.1 (cont.) The list of chemicals and enzymes used

Names	Formula	Suppliers
D-fructose	$C_6H_{12}O_6$	Ajax FineChem, Seven Hills, Australia, Cat. No. A775
Glucose (*D*-) anhydrous	$C_6H_{12}O_6$	Ajax FineChem, Wellington, New Zealand, Cat. No. A783
Lactate dehydrogenase (LDH)	-	Roche, Mannheim, Germany, Cat. No. 10 127 876 001
Magnesium sulphate, hepta hydrated	$MgSO_4.7H_2O$	Ajax FineChem, Seven Hills, Australia, Cat. No. A302
Nicotinamide adenine dinucleotide, reduced form, disodium salt (NADH)	$C_{21}H_{27}H_7O_{14}P_2Na_2$	Roche, Mannheim, Germany, Cat. No. 10 128 023 001
Octanol (1-octanol)	$C_8H_{18}O$	Panreac, Barcelona, Spain, Cat. No. 163386
Potassium dihydrogen orthophosphate	KH_2PO_4	Ajax FineChem, Seven Hills, Australia, Cat. No. A391-500G
Potassium hydroxide	KOH	RCI Labscan, Patumwan, Bangkok, Cat. No. K2002
Potassium metabisulphite (KMS)	$K_2S_2O_5$	N/A
Propan – 1 – ol (Propanol)	$CH_3CH_2CH_2OH$	Labscan Asia, Bangkok, Thailand, Cat. No. A 3543

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Table 3.1 (cont.) The list of chemicals and enzymes used

Names	Formula	Suppliers
Ronozyme A (CT)	-	DSM Nutritional Products, Product code. 04 8563 2
Ronozyme VP (CT)	-	DSM Nutritional Products, Product code. 04 8553 5
Ronozyme WX	-	Roche Vitamins Ltd., CH – 4070 Basel, Switzerland
Sodium acetate	$NaC_2H_3O_2$	Merck, Darmstadt, Germany, Product No. 1062811000
Sodium hydrogen carbonate	$NaHCO_3$	Ajax FineChem, Wellington, New Zealand, Cat. No. A475
Sodium hydroxide pellets	NaOH	Labscan Asia, Bangkok, Thailand, Cat. No. K2001
Sodium pyruvate	$C_3H_3NaO_3$	Fluka, St. Louis, USA, Cat. No. 15990
Sucrose	$C_{12}H_{22}O_{11}$	Ajax FineChem, Seven Hills, Australia, Cat. No. A530
Sulphuric acid	H_2SO_4	Labscan, Dublin, Ireland, Cat. No. A8302
Thiamine pyrophosphate (TPP)	$C_{12}H_{19}ClN_4O_7P_2S$	Sigma, St. Louis, USA, Cat. No. C8754
Triethanolamine hydrochloride	$C_6H_{15}NO_3.HCl$	Sigma, St. Louis, USA, Cat. No. T1502

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3.4 Preparation of dried longan extract and dried longan flesh powder

3.4.1 Dried longan extract

Dried longan extraction was prepared by immersing 30.0 g of dried longan in 100 ml of boiling distilled water (30%(w/v)) for 30 min (Achavasamit and Leksawasdi, 2009; Agustina *et al*., 2009). Solid was removed by filtration procedure. The dried longan extract was frozen at -20°C before used.

3.4.2 Dried longan flesh powder

Insoluble solids, which were separated from dried longan extract medium preparation, was dried by a tray dryer (Armfield, Model No. UOP8, Ringwood, United Kingdom) at 65°C for 48 h. The dried product was then crushed with hammer mill (Armfield, Model No. FT2 - P, United Kingdom).

3.5 Buffer

3.5.1 Triethanolamine buffer

Triethanolamine – HCl	250	mM
EDTA – disodium salt	25	mM

The pH was adjusted to 7.6 with 5 M NaOH

3.5.2 NADH buffer

NADH – disodium salt	6.35	mM
$NaHCO_3$	120	mM

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3.6 Equipments

Table 3.2 The list of equipment used

Equipment	Brand
Aquarium air pump	Bigboy 6000
Centrifuge machine	Nuve Bench – top centrifuge, Ankara, Turkey, Model No. NF 200
Cuvette (1.5 ml)	PlastiBrand, Wertheim, Germany, Cat. No. 759015
Double – beam spectrometer	PerkinElmer Instruments, Shelton, USA, Model No. 25 UV/Vis Spectrometer
Fume cupboard	Toplab, Thailand
Gas cooker	Lucky Frame, LF – 402, Samutprakan, Thailand
PTFE (polytetrafluoroethylene) membrane with 0.2 µm pore diameter	Midisart ® 2000, Sartorius Stedim
Hammer mill	Armfield, Model No. FT2 – P, UK
Hand refractometer	ATAGO, Tokyo, Japan, Model No. 1- α (Brix 0 – 32%)

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Table 3.2 (cont.) The list of equipment used

Equipment	Brand
High Performance Liquid Chromatography (HPLC)	Agilent Technologies, Santa Clara, California
• Autosampler	• Product No. G1313A
• Column Thermostat	• Product No. G1316A
• Diode Array Detector	• Product No. G1315A 1200 Series
• Refractive Index Detector	• Product No. G1362A
HPLC Column Altima C8, 5 μm particle size	Alltech, Lexington, Kentucky (4.6 mm inner diameter, 1 cm length)
HPLC Column Aminex HPX – 87H Ion Exclusion, 9 μm particle size	BioRad, Hercules, California (7.8 mm inner diameter, 30 cm length)
Incubator	Accuplus, Model i250, Bangkok, Thailand
Magnetic stirrer	Model No. MGS – 1001, Tokyo, Japan
Orbital shaking incubator	n – Biotek, GyeongGi – Do, Korea, Model No. NB - 205
Oven	Memert, Model No.400, Oxford, United Kingdom
pH meter	Eutech Instruments, Model pH 510, Nijkirk, Japan

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Table 3.2 (cont.) The list of equipment used

Equipment	Brand
Portable pressure sterilizer	All American, Model No. 1925x, Wisconsin, United States of America
Silicone tube	Dura, Code: EZ – TG101 (3 × 5 mm diameter), Code: EZ – TG108 (7 × 11 mm diameter)
Temperature controlled centrifuge machine	Sorvall (Thermo Fisher Scientific, Massachusetts, USA, Model No. Super T21) SL – 250T
Vortex mixer	LMS, Model VTX – 3000L, Tokyo, Japan

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3.7 Selection of suitable digestion method

3.7.1 Commercial enzyme addition strategy

Dried longan flesh powder, which was prepared from section 3.3.2, was mixed in 3:10 (w/v) ratio with 0.06% (w/v) sodium acetate. The pH level was adjusted to 5.0 by addition of 2% (w/v) acetic acid (Yoon *et al*., 2005). The suitable addition strategy was selected for three commercial enzymes, namely, α - amylase, carbohydrase and endo-xylanase using 0.17 g/g dried longan powder (enzyme activity of 100 kNU (kilo – Novo α-amylase unit), 25 FBG (fungal beta-glucanase unit) and 500 FXU (fungal xylanase unit) and 0.33 g/g dried longan powder (enzyme activity of 200 kNU, 50 FBG and 1000 FXU, respectively) (Table 3.3, adapted from Karnchanata *et al*., 2008 and Ruenprachan *et al*., 2002) with/without a pressurized steamer (All American, Model No. 1925x, Wisconsin, United State of America) at 121°C for 30 min before cooling down to room temperature. The digestion was performed in an orbital shaker incubator at 200 rpm at 50°C for 24 h (Yoon *et al*., 2005). The suitable addition strategy for commercial enzymes, the enzyme activity and using/non - using a pressurized steamer were selected for the experiment in section 3.7.2.

Table 3.3 The enzyme activity

Type of Commercial Enzyme	Enzyme activity when used enzyme mass fraction of	
	0.17 g/g	**0.33 g/g**
1. α - amylase	100 kNU	200 kNU
2. carbohydrase	25 FBG	50 FBG
3. endo-xylanase	500 FXU	1000 FXU

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Sugars (glucose, fructose and sucrose) and acetic acid concentration was analyzed by HPLC with refractive index detector and 20 min of run time. The column used was Aminex ®HPX-87H Ion Exclusive 9 μm particle size with 5 mM H_2SO_4 in distilled water. Flow rate was set at 0.75 ml/min. Temperature of column oven was set at 37°C. The refractive index was measured with Refractive Index Detector with 20 min run time. Volume of injected sample was 20 μl.

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3.7.2 Effects of digestion mixture ratio

The digestion of low sugar dried longan flesh was performed by the mixture of acetic acid and sodium hydroxide solutions. Four levels of acetic acid solution concentration (0.5, 1.0, 1.5 and 2.0%(w/v)) were mixed with 0.1%(w/v) sodium hydroxide solution with the volume ratio of 1:2, 1:1 and 2:1 to form the digestive solvents. Four ratio of dried longan flesh powder to digestive solutions of 1:10, 2:10, 3:10 and 5:10 were subsequently investigated. The pH level was adjusted to 5.0 by addition of 2%(w/v) acetic acid (Yoon *et al*., 2005). The digestion was performed in an orbital shaker incubator at 200 rpm at 50°C for 24 h. The similar digestion conditions as Section 3.7.1 were applied. The appropriate mass/volume ratio of dried longan flesh and acetic acid solution/sodium hydroxide solution mixture, acetic acid solution concentration and ratio of acetic acid solution/sodium hydroxide solution mixture were selected for digestion of dried longan flesh with low sugar level. The digested dried longan flesh hydrolysate in this study will be used as an alternative carbon source for the ethanol production microorganisms in section 3.8.

Sugars (glucose, fructose and sucrose) and acetic acid concentration was analyzed by HPLC with refractive index detector and 20 min of run time. The column used was Aminex ®HPX-87H Ion Exclusive 9 μm particle size with 5 mM H_2SO_4 in distilled water. Flow rate was set at 0.75 ml/min. Temperature of column oven was set at 37°C. The refractive index was measured with Refractive Index Detector with 20 min run time. Volume of injected sample was 20 μl.

3.7.3 Statistical analysis

The errors were calculated from 5 replicates. The statistic analysis were made by hypothesis testing method (Skoog *et al*., 1996) and hypothesis testing program for statistically significant difference (NLST_Diff version 1.0) with symbol W 1.3281 (Leksawasdi, 2009).

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3.8 Determination of growth kinetics

The fed batch cultivation of *S. cerevisiae* TISTR 5606, *C. utilis* UNSW 709400 and 709700 (Agustina *et al*., 2009) were initiated as preseed cultivation in ammonium sulphate medium, which was consisted of ammonium sulphate 8.57 ± 0.57 g/l (Maneetong *et al*., 2010; Yawiang *et al*., 2010) with total volumes of 15 ml for 18 h (Appendix E). All cultivations were done at 30°C (Laluce *et al*., 2009). The seed inocula were achieved by diluting 10% v/v inocula with corresponding volumes of dried longan extract medium until the final volume of seed inocula reached the level of 150 ml and cultivated in the same condition and incubation period (Chaweekunlayakun *et al*., 2010).

The fed batch cultivation in 5,000 ml scale was carried out in 20 l high density polyethylene drums as shown in Figure 3.1. Each drums was washed thoroughly with hot water for 5 min prior to soaking for 24 h with 200 ppm potassium metabisulphite (KMS, Wechavit), which was filled to the fullest level of the drum. The replacement of KMS solution in the drum with 4,950 ml dried longan extract medium and 1% v/v seed inoculum (50 ml) was performed aseptically (Chaweekunlayakun *et al*., 2010). The cultivation medium with 4.28 ± 0.29 g/l nitrogen source were used in the ammonium sulphate medium. This was refered to a method described by Inta *et al*. (2010) who investigated ethanol production kinetics of *S. cerevisiae* TISTR 5606 in 100 liters scale using three levels of ammonium sulphate as a nitrogen source at 4.28 ± 0.29 g/l, 8.57 ± 0.57 g/l and 17.14 ± 1.15 g/l under aeration for 12 h and without aeration for another 24 h. and 30°C. The cultivation was conducted under aeration for 12 h and without aeration for another 36 h (total cultivation time of 48 h) at 30°C. Five replicates were collected of 10 ml at each time point (0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 42, 45 and 48 h).

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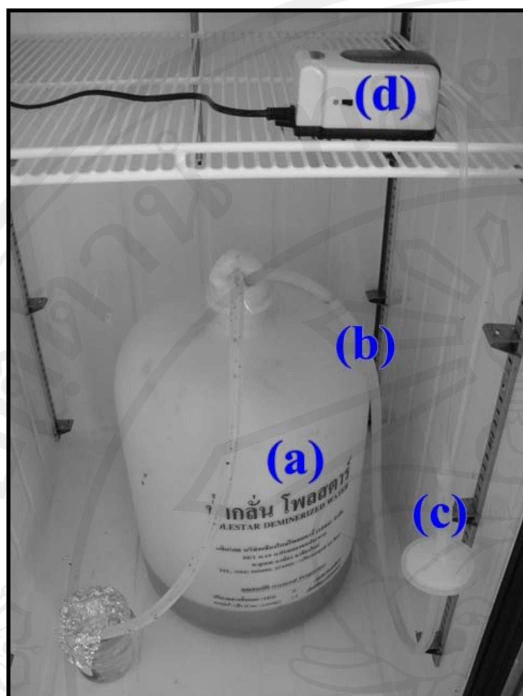

Components:

(a) 20 l high density polyethylene drums

(b) Silicone tube

(c) Air filtration housing with PTFE membrane (0.20 μm pore diameter)

(d) Aquarium air pump

Figure 3.1 Aerated fed batch system for cultivation of microbes.

The microbe growth in the sample was retarded by 4°C ice/water mixture for 5 min and kept frozen at -20°C pending subsequent analyses.

The sample was kept at 48 h of fermentation process of 500 ml. The culture was then separated into two portions of 1,000 ml. The first bottle was fed with 500 ml dried longan extract medium (initial sugars: 74.8 ± 0.7, 26.4 ± 0.2, and 33.5 ± 0.1g/l for sucrose, glucose and fructose, respectively) while the other was fed with 500 ml digested dried longan flesh hydrolysate medium which were digested by the appropriate conditions obtained from section 3.7 as the carbon source. DDLFH medium obtained from the previous digestion experiment was concentrated to acheive the initial TSS values of 20 (initial sugar: 12.5 ± 0.2 g/l sucrose, 21.9 ± 0.2 g/l glucose and 22.2 ± 0.3 g/l fructose).

The cultivations in both bottles were contained until 60 h and the five replicates sampling was scheduled on a regular interval of 3 h starting from 48 h for the period of 12 h (48, 51, 54, 57 and 60 h) at 30°C. The microbe growth in the sample was retarded by 4°C ice/water mixture for 5 min and kept frozen at -20°C

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pending subsequent analyses. Sampling was performed at 60 h of fermentation from each bottle for biotransformation process.

The supernatant and the cell pellet were separated by centrifugation at 2,822 × g (5,000 rpm) for 15 min. The supernatant obtained was analyzed for pH level by using pH meter, TSS by hand refractometer, sugars (glucose, fructose and sucrose), acetic acid and ethanol was analyzed by HPLC with refractive index detector and 20 min of run time. The column used was Aminex ®HPX-87H Ion Exclusive 9 μm particle size with 5 mM H_2SO_4 in distilled water. Flow rate was set at 0.75 ml/min. Temperature of column oven was set at 37°C. The refractive index was measured with Refractive Index Detector with 20 min run time. Volume of injected sample was 20 μl. The pellet obtained was washed once with distilled water, followed by centrifugation at 2,822 × g for 15 min. The washed cells pellet was analyzed for dried biomass weight, optical density at 600 nm.

The errors were calculated from 5 replicates which collected from each time point. The statistic comparison was made by the same hypothesis testing as section 3.7.3.

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3.9 The comparison of phenylacetylcarbinol production by whole cells from three microbial strains

The experiment of two phases emulsion system for PAC biotransformation was conducted by adopting whole cells of *S. cerevisiae* TISTR 5606, *C. utilis* UNSW 709400 and 709700 cultivated in three conditions, namely, the batch cultivation with dried longan extract at 48th h of fermentation period, fed batch cultivation with DLE and DDLFH at 60th h of fermentation period. The whole cells concentration were adjusted to dried biomass equivalent level of 12.24 g/l. The experiment was done with 5 replicates (overall experimental design: 3 (microbial strains) x 3 (conditions) x 5 (replicates) = 45 members). Because each member contained two phases, the overall analysis would be 45 x 2 = 90 analyses.

The condition for biotransformation reaction was as following. The organic phase contained 1.75 M benzaldehyde in octanol with the total volume of 5 ml. The aqueous phase of equal volume consisted of 300 mM sodium pyruvate, 1 mM thiamine pyrophosphate (TPP) and 1 mM $MgSO_4.7H_2O$ in 1.2 M phosphate buffer. After addition of whole cells, each biotransformation bottle was placed in a rotary tumbler mixer (Fabix,)at 10 rpm, 4^oC for 72 h as shown in Figure 3.2.

The microbial growth in the collected sample was slowed down by immersion in 4°C ice/water mixture for 5 min and kept frozen at -20°C pending subsequent analyses.

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Figure 3.2 The biotransformation bottle was placed in a rotary tumbler mixer at 10 rpm, $4^{o}C$ for 72 h

The phases obtained were analyzed for pH level after 72 h of reaction by pH meter, volume of each layer after centrifugation to calculate the volume ratio, PAC, benzaldehyde and benzoic acid were determined by using HPLC with diode array detector (DAD) and ultra violet (UV) detection wavelength at 263 nm for benzyl alcohol and 283 nm for other species with 20 min of run time. The column used was Altima™ C8 5 µm particle size with 32%(v/v) of acetonitrile and 0.5% (v/v) of acetic acid in distilled water. Flow rate was set at 1.0 ml/min. Temperature of column oven was set at room temperature. Volume of injected sample was 5µl as described by Rosche *et al*. (2001). Acetoin was analyzed by using HPLC with HPX-87H column. Pyruvate concentration was determined spectrophotometically by enzymatic NADH + H^{+} coupled assay method with LDH based on the modified method from Czok and Lamprecht (1974) (Appendix H). Acetaldehyde concentration was determined in the same manner as pyruvate concentration with the replacement of LDH with ADH based on modified method from Bernt and Bergmeyer (1974) (Appendix I).

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