CHAPTER 3

MATERIALS AND METHODS

3.1 SOURCES OF MICROORGANISMS

3.1.1 SAMPLES

Alkaline lipolytic bacteria were isolated from forty soil samples and five water samples obtained from various sources such as vegetable oil manufacturing industries, oil contaminated soils, dump, municipal wastes and natural resources. The samples were collected sequentially during April 1998 to October 1999 (full of gratitude to everyone who kindly collected those samples are sincerely given here). The detail of samples was shown in **APPEXDIX A.**

3.1.2 BACTERIAL REFERENCE STRAINS

Bacterial reference strains used in this study were obtained from Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok. They are *Pseudomonas sp.* and *Bacillus sp.*, which were accommodated to confirm identification of selected bacterial strains.

3.2 RAW MATERIALS AND SOURCES

Locally pure corn oil (Mazola, Samuthprakarn, Thailand) was used as substrate in the isolation of alkaline lipase producing bacteria and the commercial olive oil (Bertolli, Luca, Italy) was used as the main substrate in lipase production. Moreover, purified olive oil (by method of Jensen

et. al., 1967) was consumed in quantitation of lipase production by all bacterial isolates on rhodamine B plate. Also, other lipids, i. e., castor oil (Vidhayasom, Bangkok, Thailand), soybean oil (Thai vegetable oil, Nakhonphatom, Thailand) and palm oil (Jade brand, Lamsoong, Samutphrakan, Thailand), were used as specific substrates. The composition of those oils were:

Types of oils	Unsaturated fatty acids (%)	Saturated fatty acids (%)
Soybean oil	83.3	16.7
Corn oil	57.1	14.3
Olive oil	85.7	14.3
Castor oil	54.2	14.3
Palm oil	85.7	14.3

3.3 CHEMICALS, REAGENTS AND INSTRUMENTS

3.3.1 CHEMICALS AND REAGENTS

All chemicals used in this study were analytical grade:

Ammonium chloride, NH_4CI (Merck, Darmsatadt, Germany);

Calcium chloride, $CaCl_2$ $2H_2O$ (Merck);

Copper (II) acetate (Carlo Erba, Italy);

Copper sulfate $CuSO_4$ $5H_2O$ (Merck);

Glycine (Merck);

Iodine (May and Baker, Dagenham, England);

Magnesium sulfate, $MgSO_4$ $7H_2O$ (Merck);

Manganese chloride $MnCI_2$ $4H_2O$ (Merck);

Nikel sulfate$NiSO_4$ $5H_2O$ (Mayer and Baker);

p–nitrophenol (pNP; Fluka, Switzerland);

p–nitrophenyl laurate (Sigma, Steinheim, Germany);

Polyvinyl alcohol (Carlo Erba).

Potassium dihydrogen phosphate KH_2PO_4 (Merck);

Potassium iodide KI (Merck);

Sodium acetate CH_2COONa (Merck);

di-Sodiumhydrogen phosphae Na_2HPO_4 (Merck);

Sodium hydroxide NaOH (Merck);

Sodium molybdate Na_2MoO_4 (Merck);

Sodiumselenate Na_2SeO_3 (Merck);

Sodium tungstate, Na_2WO_4 (Mayer and Baker);

Zinc chloride $Zncl_2$ $2H_2O$ (Merck).

Reagents used in this study were given as following:

Hydrochloric acid HCI (Merck);

Isooctane (Carlo Erba);

0.85% Normal Saline (Merck);

Pure oleic acid (Carlo Erba);

Pyridine (Carlo Erba);

Sodium hydroxide 4N NaOH (Merck);

Triton X–100 (USB Chemicals, Buckinghamshire, England);

Tween80 (Merck);

Gram Staining dyes, namely;

Crystal violet (Merck);

95% ethyl alcohol (Merck);

Rhodamine B (Fluka); and

Safanin 0 (BDH Chemical, Poole, England).

3.3.2 INSTRUMENTS

Many instruments were used, for example;

Autoclave (Labo Autoclave, Sanyo Electric, Japan, grateful thanks to Viriya Insurance, Co., Thailand, for donation of two sets to Department of General Science, Faculty of Science, Chulalongkorn University);

High–Speed Centrifugation (ALC Model 4239 R, Italy);

Incubator (Memmert GmbH, Model 700, Schwabach, Germany);

Microwave (Sanyo electric, Japan, grateful thanks to Thanes, Co., Thailand for donation to the Department of General Science);

Oven (Memmert GmbH, Model 700, Schwabach, Germany).

pH meter (Model pH scan1, Singapore);

Refrigerator (Samsung, Korea, grateful thanks to Becthai, Co., Thailand for donation to the Department of General Science);

Rotary Shaker (Lab–Line Instrument, Melrose Park, Illinois, U.S.A.); and

Spectrophotometer(Spectronic^{S} 20 Genesys^{TM}, U.S.A.);

3.4 CULTURE MEDIA

3.4.1 GENERAL MEDIA

The formulas and preparation of each culture medium were shown in **APPENDIX B.** Those were:

Nutrient Broth (NB; Difco laboratories, Detroit, Michigan, U.S.A.); and

Nutrient Agar (NA; Difco)

3.4.2 SELECTIVE MEDIA

For identification of the selected alkaline lipase producing bacteria, some selective media were used, for example,

Shigella–Salmonella Agar (SSA; Difco);

MacConkey Agar (MA; Difco);

Pseudomonas Selective Isolation Agar (PSIA, Difco);

Eosi-Methylene Blue Agar (EMB; Difco); and

Triple Sugar Iron (TSI, Difco)

3.4.3 MEDIA FOR ALKALINE LIPASE PRODUCING BACTERIA

3.4.3.1 Medium for Screening and Isolation

Lipid–Rhodamine B Agar plate (LRA) was the semisolid medium. Modified Lipid–Rhodamine B Agar plate was used instead of

the originally one (Wang et. al., 1995), i. e., lack of yeast extract. Fluid medium for isolation of alkaline lipase producing bacteria is lipid broth (LOB) with yeast extract but no agar and rhodamine B. Trioleoylglycerol Rhodamine B agar plate (TRA) was also used as medium for screening of bacteria with high alkaline lipase production.

3.4.3.2 Medium for Cultivation and Measurement of Bacterial Growth

Lipase Production Medium (LPM) was liquid medium prepared according to method of Schmidt–Dannert et. al. (1994). Measurement of Growth Medium (MGM) was also liquid medium described by Wang et. al. (1995).

All formulas and preparations of each medium were illustrated in **APPENDIX B.**

3.5 STAINING AND BIOCHEMICAL TESTS FOR IDENTIFICATION

Formula, preparation and procedure of staining and some biochemical tests were illustrated in **APPENDIX C.** Gram's Stain and Indian Ink was prepared. For examining of capsule and endospore forming in bacteria, the Indian Ink was also used.

Some biochemical test were performed such as Triple Sugar Iron (TSI) Agar, Motility Test, Oxidation–Fermentation Test, Citrate Utiligation, Catalase Test,Oxidase Test, Acetate Utilization, Hydrolysis of starch, Hydrolysis of casein, Hydrolysis of gelatin and Urease Test.

3.6 BACTERIOLIGICAL PROCEDURES*

3.6.1 SAMPLING AND CULTIVATION PROCEDURES

3.6.1.1 Sampling

Soil and wastewater samples were obtained from certain areas where have been contaminated by oil as well as hydrocarbons and from natural sources. Two replicates of soil and water samples were collected at each sampling site (500 g, and/or 500 ml for one replicate). All samples were stored in ice box to laboratory and then kept in refrigerator. Isolation and screening of alkaline lipase producing bacteria were conducted either in the same or after day of collection.

3.6.1.2 Screening and Isolation of Alkaline Lipase producing bacteria

Each of soil samples as well as water samples was suspended and performed serial dilutions with normal saline (8.5 $g/_L$ NaCl); and then spread on LRA plates (pH 9.0) prepared previously. After that the plates were incubated at 37°C for up to 48 hours, each isolated colony surrounded with an orange fluorescent halo upon ultraviolet radiation at 350 nm, regarded as lipase producers, was inoculated in LOB at 37°C for 24 hours. Later, the culture was recultivated, by streaking on LRA plate.

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*All chemicals, reagents, cultivation media, glasswares and certain devices were sterilized by autoclaving at 121 °C for 15 minutes; and at least three replicates were conducted for each test.

A single colony was cultivated at least 2 times to assure the purity of culture and then stabbed in the stock culture tubes containing corn oil and NA. Those pure cultures of the probably alkaline lipase producing bacterial isolates were further proceeded.

3.6.1.3 Selection of Highly Alkaline Lipase Producing Bacteria by Specific and Sensitive Plate Assay (Kouker and Jaeger, 1987)

All bacterial isolates were tested for high concentration of alkaline lipase production on Trioleoylglycerol Rhodamine B Agar or TRA plates. This technique would be carried step by step as follow:

i) Punched 5 mm–diameter holes into the agar.

ii) filled holes with 30 µl of cell–free culture supernatant which came from centrifuged cell culture after cultivation at 37°C for 24 hours.

iii) incubated at 37°C for 20 hours.

iv) monitored by irradiating plates with UV light at 350 nm.

Those bacterial isolates clearly exhibited their higher lipase activity from cell-free supernatant by appearance of a larger orange fluorescent holo upon irradiation of UV light at 350 nm. They were chosen as the selected alkaline lipase bacterial strains. Then those selected strains were again tested to confirm their lipase activities by spectrophotometric method, which was described in 3.8.1.

3.6.1.4 Identification of the Selected Bacterial Strains

Five of 350 bacterial isolated were found to produce higher amounts of lipase than others. However, by spectrophotometric examination on their lipase activity, it was clearly found that only two highest alkaline lipase producing bacteria of the five selected bacterial isolates should be further identified. Each isolate was tested for some basically taxonomic characteristics, i.e., size, color and appearance of colony; gram staining and morphology of the cells (size, shape and spore formation) by microscopic method. Furthermore, Electron–microscopic was also recommended. Expectation of genus was performed primarily, by growing of the selected bacterial strains on selective media. Later, they were ascertained by some biochemical tests, compared with bacterial reference stains.

3.6.2 EFFECTS OF SOME ENVIRONMENTAL FACTORS ON GROWTH OF THE SELECTED BACTERIAL STRAINS

3.6.2.1 Effect of pH

Two selected bacterial isolates were cultivated individually in 250-ml conical flask containing Lipase Production Medium LPM. The pH values of the culture medium were adjusted to be 5 to 11 by adding 0.1 N HCl for acidic pH range and 0.1 N NaOH for alkaline pH range. Each pH value was maintained with 1.0 M buffer system (phosphate buffer pH 5–6; Tris–HCl pH 7–9; glycine–NaOH pH 10–11). The certain

amounts of overnight culture were inoculated in sterilized LPM in each culture flasks described above, and then incubated at 37°C for 24 hours. The viable count method was conducted to determine the number of each tested organism. The highest amount of the tested bacterial isolate was observed in medium adjusted to certain pH value.

3.6.2.2 Effect of Temperature

The procedure for study the temperature influenced on growth of selected bacterial strains was performed at different temperature. However, the pH value of medium used was optimum pH value that was obtained from study on the effect of pH in 3.6.2.1 for each organism. The certain amounts of the test bacterial strains were inoculated into sterile LPM in each culture flask and then incubated for 24 hours at various temperatures ranging from 25 to 65°C. The number of each tested bacterial isolate was determined by viable count method. The highest amount of the tested bacterial strain was found in medium incubated at certain temperature of incubation.

3.6.3 GROWTH CONDITIONS OF THE SELECTED BACTERIAL STRAINS

Determination of growth conditions for the lipase production, some parameters were studied such as effect of nitrogen sources, pH of medium and the lipid substrates according to Wang et. al. (1995). Certain amount of inoculum that was prepared by cultivation of each organism for 20 hours in 250- ml flask containing 50 ml of LOB and

incubating at 37°C on rotary shaker (200 rpm) was aseptically transferred to individual conical flask containing MGM with optimum pH value for each strain and again incubated at appropriate temperature, previously studied in 3.6.2.1 and 3.6.2.2). Growth of each bacterial strain was directly measured by viable count method.

3.7 CHEMICAL ANALYSIS PROCEDURES

3.7.1 EFFECT OF DIFFERENT MEDIA ON LIPASE PRODUCTION

Effect of each medium on Lipase production of the selected strains was carried out in two kinds of liquid media with optimum pH value for those strains, one was LOB and the other was LPM, by inoculating separately each overnight culture of selected organisms with 2% inoculum size in those media. Later the cultivation on shaker at 200 rpm was performed at optimum temperature, which previously was studied in 3.6.2.2, for each isolate. The cell-free supernatants from cell cultures of the selected bacteria were obtained by centrifugation at 9,700 rpm for 20 minutes at 4°C. Then lipase activity in each supernatant from selected strains was measured by using spectrophotometric method discussed in 3.8.1.

3.7.2 PREPARATION OF LIPASES

Similar to procedure in 3.7.1, each overnight culture in NB of the selected bacterial strains was individually transferred into 500-ml Erlenmeyer flasks containing 100 ml of LPM with optimum pH as well as 2% inoculum and cultivated on rotary shaker (200 rpm) at optimum temperature for each strain. The cell cultures in stationary growth phase were harvested. After that the cells from both culture broth were removed by centrifugation at 9,700 rpm for 20 minutes at 4°C. Both supernatants with crude lipases of the selected bacterial isolates were collected and kept at 4°C for further study, i. e., characterizations of the enzymes.

3.8 ASSAY FOR THE LIPASE ACTIVITY

Lipase was routinely analyzed spectrophotometrically by monitoring the p–nitrophenol released from p–nitrophenyl laurate as the substrate according to a method described by Winkler and Stuckmann, 1979, see 3.8.1. Because of unstability of p–nitrophenyl laurate at a basic pH, the cupric acetate method was conducted for activity assays in the determination of lipase's pH stability and optimum pH by measuring the free fatty acids released from olive oil (Kwon and Rhee, 1986, see 3.8.2). Moreover, the estimated quantity of lipase production was assayed by specific and sensitive plate method as mention above.

3.8.1 MEASURING OF RELEASED p–NITROPHENOL (WINKLER AND STUCKMANN, 1979)

The liberated p–nitrqohenol from the reaction mixture was measured spectrophotometrically. The process would be conducted in serial steps as follows:

i) placed 0.5 ml of substrate solution (8% p-nitrophenyl laurate and Triton X-100 in 5mM sodium acetate buffer, pH 5.0) in test tube.

ii) added 0.5 ml of 50 mm Tris – HCl buffer.

iii) added 50 µl of appropriately diluted enzyme solution

iv) mixed thoroughly.

v) incubated at 37°C for 15 minutes.

vi) measured the released p–nitrophenol by reading absorbance at 410 nm.

One unit of enzyme activity was defined as the amount of enzyme required to release 1 nmol of p–nitrophenol per min under the described condition. The sample was done by, at least, three replicates. Preparation of reagents and the standard curve as well as calculation the unit of enzyme activity, were described in **APPENDIX D.**

3.8.2 DETERMINATION OF FREE FATTY ACIDS (KWON AND RHEE, 1986)

The released free fatty acids were also determined by spectrophotemetric method. The procedure of quantifying free fatty acids

would be performed sequentially as follows after stopping the reaction mixture:

i) added 3 ml of isooctane to stopped reaction.

ii) mixed with vortex for at least 2 minutes.

iii) centrifuged at 1,400 rpm for 5 minutes.

iv) drawn off 1.5 ml of isooctane layer containing free fatty acids and then mixed with 1 ml of cupric acetate–pyridine reagent.

v) vortexed thoroughly.

vi) measured the upper layer by reading absorbance at 715 nm.

One unit of lipase is defined as the amount of enzyme which liberate 1 nmol of free fatty acids per minute under the assay conditions. The sample was performed by three replicates. Preparation of reagents and standard carve using oleic acid, as well as calculation the unit of enzyme activity, were given in **APPENDIX E.**

3.9 EFFECT OF SOME ENVIRONMENTAL FACTORS ON ACTIVITY OF CRUDE LIPASES

3.9.1 EFFECT OF pH

The effect of pH on lipase activity was performed as following. 100 µl of crude lipase produced individually from each of the selected bacterial isolates was added to each reaction mixture tube containing emulsion of 0.5 ml of olive oil and 0.5 ml of 100 mM various buffer solutions with pH value ranging from 3 to 11 (glycine–HCl pH 3–

4; phosphate buffer pH 5–6; Tris–HCl pH 7–9; glycine–NaOH pH 10–11). The reaction mixture was incubated at 37°C. After 1 hour incubation, the reaction mixture was stopped with 2 ml of 6N HCl, then lipase activity in each tube was assayed according to method described in 3.8.2. Each pH value of reaction mixture was done with, at least, three replicates.

3.9.2 pH Stability

Similarity to effect of pH, the pH stability of enzymes of selected strains was also separately performed. Each of 100 µl of crude lipase was added to the test tube containing 0.5 ml of 100 mM various buffer solutions as the same as in pH–influence experiment without olive oil. After that those tubes were incubated at 5°C for 24 hours, and lipase activity was analyzed by adding 0.5 mve oil in each tube, mixing thoroughly, incubating at 37°C for 1 hour and stopping the reaction mixture with 2 ml of 6N HCl, respectively, according to 3.8.2.

3.9.3 EFFECT OF TEMPERATURE

Test concerned effect of temperature on lipase activity of the selected organisms was carried out by using p–nitrophenyl laurate as a substrate for lipase activity, the method was described in 3.8.1. The mixture was incubated at different temperature range, i. e., 15, 25, 35, 45, 55, 65, 75 and 85°C. After incubation, released p–nitrophenol was measured spectrophotometrically to determine the activity of lipases from the selected isolates.

3.9.4 THERMAL STABILITY

Thermostability test of crude lipases from each strain was done by adding 50 µl of appropriately diluted enzyme to each test tube containing 0.5 ml of 50 mm Tris–HCl pH 8.5 and then incubated at various temperature, ranging from 15 to 85°C, for 20 minutes, 0.5 ml of substrate solution was added in enzyme solution tubes and mixed thoroughly. After 15 minutes of incubation at 37°C, the reaction mixture was read optical density at wavelength 410 nm, according to method described in 3.8.1, to measure lipase activity.

3.10 DETERMINATION OF SUBSTRATE SPECIFICITY

The substrate specificity test of crude lipase from the selected bacteria was performed by substitution of 0.5 ml of olive oil with other oils such as soybean oil, corn oil, castor oil and palm oil. The procedure was that lipase enzyme was added into emulsified reaction mixture containing 0.5 ml of 110 mM of buffer solution with appropriate pH value and incubated at optimum temperature of individual crude lipase from selected bacterial strain as previously observed in 3.9.1and in 3.9.3 for 1 hour. Consequentially, the activity of lipase was measured after adding 2 ml of 6N HCl, according to the assay method described previously in 3.8.2.