REFERENCES

ลาวัณย์ ไกรเดช. 2519. เทมเป้และผลิตภัณฑ์เลียนแบบเทมเป้. **อาหาร.** 8(3): 21-28.

วัฒนาลัย ปานบ้านเกร็ด และ สรวง อุดมวรภัณฑ์. 2536. **เทคนิคทางอณูพันธุศาสตร์และพันธุวิศวกรรม.** นครปฐม : โรงพิมพ์สาธารณสุขมูลฐานอาเซียน.

Akeson, W.R., and Stahmann, M.A. 1964. A pepsin-pancreatin digest index of protein quality. **J. Nutr.** 83: 257.

Akimoto, T., Matsumoto, I., and Imai, S. 1993. Effect of temperature and inoculum size on enzyme activities during natto fermentation. **J. Jpn. Soc. Food Sci. Technol.** 40(1): 83-90.

Anderson, D.R., Sweeney, D.J., and Williams, T.A. 1981. **Introduction to statistics : An applications approach.** Minnesota: West Publishing Co.

Association of Official Analytical Chemists (AOAC). 1990. **Official Method of Analysis of the Association of Official Analytical Chemists** - 15th ed., Washington. D.C.

Batra, L.R., and Miller, P.D. 1976. Asian fermented foods and beverages. **Dev. Ind. Microbiol.** 17: 117-128.

Beuchat, L.R. 1984. Fermented soybean foods. **Food Technol.** 38(6): 64-70.

Beuchat, L.R., and Worthington, R.E. 1974. Changes in the lipid content of fermented peanuts. **J. Agric. Food Chem.** 22: 509-512.

Bodwell, C.E., Satterlee, L.D., and Hackler,L.R. 1980. Protein digestibility of the same protein preparations by human and rat assays and by *in vitro* enzymatic digestion methods. **Am. J. Clin. Nutr.** 33: 677-686.

Bol, J., and Smith, J.E. 1989. Biotransformation of aflatoxin. **Food Biotechnol.** 3: 127-144.

Borlaug, N.E. 1992. Lighting fires at grass roots. **Food Technol.** 46(7): 84-85.

Bulan Phithakpol, Suparat Reungmaneepaitoon, and Warunee Varanyanon, eds. 1995. **The traditional fermented foods of Thailand.** Kuala lumpur: SP-Mud Printing Sdn.Bhd.

Calloway, D.H., Hickey, C.A., and Murphy, E.L. 1971. Reduction of intestinal gas-forming properties of legumes by traditional and experimental food processing methods. **J. Food Sci.** 36: 251-255.

Campbell-Platt, G. 1987. **Fermented foods of the world- a dictionary and guide.** London: Butterworths.

Church. D.C. 1986. **Livestock feeds and feeding.** 2nd ed. USA: Prentice-Hall A Division of Simon & Schuster, Inc.

Circle, S.J., and Smith, A.K. 1975. Soybeans: processing and products. In N.W. Pirie (ed.), **Food Protein Sources**, pp.47. Great Britain: Cambridge University Press.

de Reu, J.C., Ramdaras, D., Rombouts, F.M., and Nout, M.J.R. 1994. Change in soya bean lipids during tempe fermentation. **Food Chem.** 50: 171-175.

de Reu, J.C., ten Wolde, R.M., de Groot, J., Nout, M.J.R., Rombouts, F.M., and Gruppen, H. 1995. Protein hydrolysis during soybean tempe fermentation with *Rhizopus oligosporus*. **J. Agric. Food Chem.** 43: 2235-2239.

Djien, K.S., and Hesseltine, C.W. 1979. Tempe and related foods. **Economic Microbiology.** 4: 115-140.

Fields, R. 1972. The rapid determination of amino groups with TNBS (Trinitrobenzenesulfonic acid), pp.464-468. In C.H.W. Hirs, and S.M. Timasheff (eds.), **Method in enzymology vol xxv enzyme structure, part B.** New York : Academic Press .

Garcia - Diaz, A., and Phillips, D.T. 1995. **Principles of experimental design and analysis.** Great Britain: T.J.Press, Padstow, Cornwall.

Gerhardt, P. 1981. **Manual of methods for general bacteriology. USA: American Society for Microbiology.** 2nd. ed. San Francisco: W.H. Freeman and Company.

Gueguen, J., and Cerletti, P. 1994. Proteins of some legume seeds: soybean, pea, fababean and lupin. In B.J.F. Hudson (ed.), **New and developing sources of food proteins**, pp 145-193. Great Britain: St. Edmundsbury Press Ltd.

Hachmeister, K.A., and Fung, D.Y.C. 1993. Tempeh: A mold-modified indigenous fermented food made from soybeans and/or cereal grains. **Crit. Rev. Microbial.** 19(3) : 137-188.

Hackler, L.R., Steinkraus, K.H., van Buren, J.P., and Hand, D.B. 1964. Studies on the utilization of tempeh protein by wealing rats. **J. Nutr.** 82: 452-456.

Hayashi, U. 1974. Study of the cause of increase in nitrogen content in the course of manufacture of natto. IV. Experiment to confirm the ability of *Bacillus natto* to utilize atmospheric nitrogen by means of stable isotopes ^{15}N. **Reports of Teikoko Joshi Daigaku Laboratory of Natto.** 49: 30-34.

Hesseltine, C.W., and Wang, H.L. 1972. Fermented soybean food products. In A.K. Smith, and S.J. Circle (eds.), **Soybean: Chemistry and Technology**. AVI Publishing Co., Westport, Conn.

Hesseltine, C.W., Smith, M., Bradle, B., and Djien, K.S. 1963. Investigation of tempeh, an Indonesia food. **Dev. Ind. Microbiol.** 4: 275-287.

Hesseltine,C.W., 1965. A millennium of fungi, food, and fermentation. **Mycologia.** 57: 149-197.

Hoag, E.H., Sarett, H.P., and Cheldelin, V.H. 1945. Use of *Lactobacillus arabinosus* 17-5 for microbioassay of pantothenic acid. **Ind. Eng. Chem. Anal. Ed.** 17,60.

Hsu, H.W., Vavak, D.L., Satterlee, L.D., and Miller, G.A. 1977. A multienzyme technique for estimating protein digestibility. **J. Food Sci.** 42(5): 1269-1273.

Iljas, N., Peng, A.C., and Gould, W.A. 1970. **Tempe find ways to preserve Indonesia soyfood.** Ohio Rep. 55: 20.

James, C.S. 1995. **Analytical chemistry of foods.** Oxford: The Alden Press.

Joint FAO/WHO Ad Hoc Export Committee. 1973. **Energy and protein requirements.** WHO Tech Rep. No. 522, Geneva. Switzerland.

Kaew Kangsadalampai, and Pongtorn Sungpuag. 1984. **Labaratory Manual for food analysis.** Bangkok: Prayurawong Co.,Ltd.

Kao, C., and Robinson, R.J. 1978. Nutritional aspects of fermented foods from chickpea, horsebean, and soybean. **Cereal Chem.** 55: 512-517.

Karyadi, D. 1985. Nutritional implications of tempeh in Indonesia rural community. **Proceedings from the Asian symposium on non-salted soybean fermentation.** Japan : Tsukuba.

Kim, B.N., Park, C.H., Yun, B.M., Jung, M.C., and Lee, S.Y. 1995. **J. Korean Soc. Food Nutr.** 24(1): 114-120.

Ko, S.D. 1974. Self-protection of fermented foods against aflatoxins. **Proceeding of the 4th International congress on Food Science and Technology.** 3: 244-253.

Ko, S.D., and Hesseltine, C.W. 1979. Tempe and related foods. In A.H. Rose (ed.), **Economic Microbiology, vol.4 : Microbial Biomass**, pp. 115-140. London: Academic Press.

Liem, I.T.H., Steinkraus, K.H., and Cronk, T.C. 1977. Production of vitamin B-12 in tempeh, a fermented soybean food. **Appl. Environ. Microbiol.** 34(6): 773-776.

Lim, G. 1991. Indigenous fermented food in south east Asia. **Asean Food J.** Vol.6, No.3, p. 83-101.

Mahmud, M.K., Herman, and Karyadi, D. 1985. A preliminary study on the use of tempeh-based formula in the dietary treatment of chronic diarrhea. **Majalah Kerehatan Indonesia.** 35: 443-446.

Mak, M.L. 1986. **Tempeh production from soybean milk wastes and soybeans by various *Rhizopus* strains.** B.Sc Hons.'s Thesis, Nat. Univ. Singapore.

Manachini, P.L., Fortina, M.G., and Parini, C. 1987. Purification and properties of an endopolygalacturonase produced by *Rhizopus stolonifer*. **Biotechnol. Lett.** 9: 219-224.

Martinelli, A.F., and Hesseltine, C.W. 1964. Tempeh fermentation: package and tray fermentation. **Food Technol.** 18: 167-171.

Matheson, N.A. 1974. The determination of tryptophan in purified proteins and in fooding - stuffs. **Br. J. Nutr.** 31: 393-400.

Meason, V.C., Bech, A.S., and Rudemo, M. 1980. Hydrolysate preparation for amino acid determination in feed constituents. **Proceeding of the 3rd E.A.A.P. Symposium.** Braurishwerg, F.R., Germany.

Mega, J.A., Lorenz, K., and Onayemi, O. 1973. Digestive acceptibility of proteins as measured by the initial rate *in vitro* proteolysis. **J. Food Sci.** 38: 173.

Mital, B.K., and Garg, S.K. 1990. Tempeh - technology and food value. **Food Rev. Int.** 6(2): 213-224.

Murata, K., Ikehata, H., and Miyamoto, T. 1967. Studies on the nutritional value of tempeh. **J. Food Sci.** 32: 580-585.

Nahas, E. 1988. Control of lipase production by *Rhizopus oligosporus* under various growth conditions. **J. Gen. Microbiol.** 134: 227-233.

Nicolas, D.J.D., 1952. The use of fungi for determining trace metals in biological materials. **Analyst.** 77: 629-642.

Nout, M.J.R. 1989. Effect of *Rhizopus* and *Neurospora* spp. on growth of *Aspergillus flavus* and *A. parasiticus* and accumulation of aflatoxin B-1 in ground nut. **Mycol. Res.** 93: 518-523.

Nout, M.J.R., and Rombouts, F.M. 1990. Recent developments in tempe research. **J. Appl. Bacteriol.** 69: 609-633.

Nout, M.J.R., Bonants-van Laarhove, T.M.G., de Dreu, R., and Gerats, I.A.G.M. 1985. The influence of some process variables and storage conditions on the quality and shelf-life of soybean tempeh. **Antonie van Leeuwenhock.** 51: 523-5334.

Nout, M.J.R., Notermans, S., and Rombouts, F.M. 1988. Effect of environmental conditions during soya-bean fermentation on the growth of *Staphylococcus aureus* and production and thermal stability of enterotoxins A and B Int. **J. Food Microbiol.** 7: 299-309.

Novelli, G.D., and Schmetz, H.J. 1951. An improved method for the determination of pantothenic acid in tissues. **J. Biol. Chem.** 192: 181.

Nutjira Imanothai. 1986. **Influence of conventional processing on vitamins and protein of different Thai foods.** Master's Thesis, Mahidol University.

Odunfa, S.A. 1985. Biochemical changes in fermenting African locust bean (*Parkia biglobos*) during "ira" fermentation. **J. Food Technol.** 2: 295-303.

Ohta, T. 1986. Natto. In N.R. Reddy, M.D. Pierson, D.K. Salunkhe (eds.), **Legume - based fermented foods**, pp. 85-93. CRC Press. Boca Raton, FL.

Osborne, D.R., and Voogt, P. 1972. **The analysis of nutrients in food**. London: Academic Press, Inc. Ltd.

Pitch-on Wana-intarayude, and Warawut Krusong. 1992. Enrichment of essential amino acid in tempeh. **Food**. 22(1): 18-23.

Poolsri Suwisuttagul. 1979. **Studies on contaminated microorganisms in some cosmetics**. Master's Thesis, Chulalongkorn University.

Pornthip Charoenthamawat. 1991. Improvement of nutritional and organoleptic quality of soy residue tempe. **Food**. 21(4): 288-295.

Ramakrishnan, C.V. 1979. Studies on Indian fermented foods, Baroda: J. Nutr. 6: 1-54.

Reddy, N.R., Pierson, M.D.,and Salunkhe, D.K, eds. 1986. **Legume-based fermented foods**. CRC Press,Boca Raton,FL.

Rhinehart, D. 1975. **A nutritional characterization of the distiller's grain protein concentrates.** Master's thesis. University of Nebraska, Lincoln, NE.

Roelofsen, P.A., and Talens, A. 1964. Changes in some B vitamins during molding of soybeans by *Rhizopus oryzae* in the production of tempeh kedelee. **J. Food Sci**. 29: 224.

Romero, J., and Ryan, D.S. 1978. Susceptibility of the major storage protein of the Bean, *Phaseolus vulgaris* L., to *in vitro* enzymatic hydrolysis. **J. Agric. Food Chem**. 26: 784-787.

Sachde, A.G., Al-Bakir, A.Y., and Sarhan, H.R. 1987. Effect of cultural conditions on the production of polygalacturonase from *Rhizopus arrhizus*. Iragi. **J. Agric. Sci**. 5: 61-72.

Sano, T. 1961. Feeding studies with fermented soy products (natto and miso). In **Meeting protein needs of infants and children**. Natl Acad, Sci-Natl. Research Counc., Washington D.C., Publ. 843, 257.

Sarkar, P.K. and Tamang, J.P. 1995. Changes in the microbial profile and proximate composition during natural and controlled fermentations of soybeans to produce kinema. **Food Microbiol**. 12: 317-325.

Sarkar, P.K., Cook, P.E., and Owens, J.D. 1993. *Bacillus* fermentation of soybeans. **World J. Microbiol. Biotechnol.** 9: 295-299.

Sarkar, P.K., Jones, L.J., Gore, W., Craven, G.S, and Somerset, S.M. 1996. Changes in soya bean lipid profiles during kinema production. **J. Sci. Food Agric.** 71: 321-328.

Sarkar, P.K., Tamang, J.P., Cook, P.E., and Owens, J.D. 1994. Kinema - a traditional soybean fermented food: proximate composition and microflora. **Food Microbiol.** 11: 47-55.

Sarwar, G. 1990. Review of protein quality evaluation methods. **J. Asso. Off. Anal. Chem.** 73: 347.

Satterlee, L.D., Kendrick, J.G., and Miller, G.A. 1977. Rapid *in vitro* assays for estimating protein quality. **Food Technol.** 31: 73.

Seeley, H.W., and van Demark, P.J. 1972. **Microbes in action: A laboratory manual of microbiology.** 2nd ed. San Francisco: W.H. Freeman and Company.

Shurtleff, W., and Aoyagi, A. 1979. **The book of tempeh.** New York: Harper and Row.

Smith, A.K., Rackis, J.J., Hesseltine, C.W., Smith, M., Robbins, D., and Booth,A.N. 1964. Tempeh: Nutritive value in relation to processing. **Cereal Chem.** 41: 173-181.

Sorenson, W.G., and Hesseltine, C.W. 1966. Carbon and nitrogen utilization by *Rhizopus oligosporus*. **Mycologia.** 58: 681.

Souser, M.L., and Miller, L. 1977. Characterization of the lipase produced by *Rhizopus oligosporus*, the tempeh fungus. **Abstr. Annu. Meet. Am. Soc. Microbiol.** 77: 258.

Steinkraus, K.H., Hwa, Y.B., van Buren, J.P., Provvidenti, M.I., and Hand, D.B. 1960. Studies on tempeh - an Indonesian fermented soybean food. **Food Res.** 25: 777-788.

Steinkraus, K.H., ed. 1983. **Handbook of indigenous fermented foods.** New York : Marcel Dekker.

Steinkraus, K.H., Hand, D.B., van Busen, J.P., and Hackler, L.R. 1961. **Proc. conf. soybean products for protein in human food,** p.75.

Stillings, B.R., and Hackler, L.R. 1965. Amino acid studies on the effect of fermentation, time, and heat processing of tempeh. **J. Food Sci.** 30: 1043.

Sudarmadji, S., and Markarkis, P. 1978. Lipid and other changes occuring during the fermentation and frying of tempeh. **Food Chem.** 3: 165-170.

Sudarmadji, S., and Markarkis, P. 1977. The phytate and phytase of soybean tempeh. **J. Sci. Food Agric.** 28: 381-383.

Taira, H., and Suzuki, N. 1983. Lipid content and fatty acid composition of natto. **Report of the National Food Research Institute** (Shokuryo Kenkyusho Kenkyu Hokoku) ; No. 43, 58-61.

Tanaka, N., Kovats, S.K., Guggisberg, J.A., Meske, L.M., and Doyle, M.P. 1985. Evaluation of the microbiological safety of tempeh made from unacidified soybean. **J. Food Prot.** 48: 438-441.

van Buren, J.P. Hackler, L.R., and Steinkraus, K.H. 1972. Solubilization of soybean tempe constituents during fermentation. **Cereal Chem.** 49: 208-210.

van der Riet, W.B., Wight, A.W., Cilliers, J.J.L., and Datel, J.M. 1987. Food chemical analysis of tempeh prepared from south African-grown soybeans. **Food Chem.** 25 : 197-206.

van Veen, A.G., and Shaefee, G. 1950. The influence of the tempeh fungus on the soya bean. **Documenta Neer. Indones. Morbis Trop.** 2: 270-281.

VELP Scientifica. 1994. **FIWE Extraction unit for determing raw fiber content operation manual.** edition September .

Wagenknecht, A.C., Mattick, L.R., Lewin, L.M., Hand, D.B., and Steinkraus, K.H. 1961. Changes in soybean lipids during tempeh fermentation. **J. Food. Sci.** 26: 373-376.

Wang, H.L. 1984. Tofu and tempeh as potential protein sources in the western diet. **J. Am. Oil Chem. Soc.** 61: 528-534.

Wang, H.L., and Hesseltine, C.W. 1965. Studies on the extracellular proteolytic enzymes of *Rhizopus*. **Can. J. Microbiol.** 11: 727-732.

Wang, H.L., and Hesseltine, C.W. 1981. Use of microbial cultures : legume and cereal products. **Food Technol.** 35: 79-83.

Wang, H.L., and Hesseltine,C.W. 1966. Wheat tempeh. **Cereal Chem.** 43: 563-570.

Wang, H.L., Ruttle, D.I., and Hesseltine, C.W. 1969. Antibacterial compound from a soybean product fermented from *Rhizopus oligosporus*. **Proc. Soc. Exp. Biol. Med.** 131: 579-583.

Wang, H.L., Ruttle, D.I., and Hesseltine, C.W. 1968. Protein quality of wheat and soybeans after *Rhizopus oligosporus* fermentation. **J. Nutr.** 96: 109-114.

Wang, H.L., Swain, E.W., Wallen, L.L., and Hesseltine, C.W. 1975. Free fatty acids identified as antitryptic factor in soybean fermented by *Rhizopus oligosporus*. **J. Nutr.** 105: 1351-1355.

Whitaker, J.R. 1978. Biochemical changes occuring during the fermentation of high-protein foods. **Food Technol.** 32: 175-180.

Winarno, F.G., and Reddy, N.R. 1986. Tempeh. In N.R. Reedy, M.D. Pierson, and D.K. Salunkhe (eds.), **Legume-based fermented foods**, pp. 95-117. CRC Press, Boca Raton, FL.

Zamora, R.G. and Veum, T.L. 1988. Nutritive value of whole soybeans fermented with *Aspergillus oryzae* or *Rhizopus oligosporus* as evaluated by neonatal pigs. **J. Nutr.** 118: 438-444.

Zamora, R.G., and Veum, T.L. 1979. The nutritive value of dehulled soybeans fermented with *Aspergillus oryzae* or *Rhizopus oligosporus* as evaluated by rats. **J. Nutr.** 109: 1333-1339.

APPENDIX I

DETERMINATION OF NUTRITIVE VALUE

1. Proximate analysis

1.1. Determination of moisture by hot-air oven method (Osborne and Voogt,1972 ; AOAC,1990 ; James, 1995)

1.1.1. Dry a coded, clean crucible in the oven for about 30 min, cool in a desiccator and weigh.

1.1.2. Mix the prepared sample thoroughly and transfer about 2 g to the crucible. Weigh the crucible and contents, as rapidly as possible

1.1.3. Dry in an oven at 101-105°C to constant weight, allowing for 6 h.

1.1.4. Remove the crucible from the oven, cool in a desiccator, and reweigh when cold.

1.1.5. Dry for a further hour period until successive weighings differ by less than 1.0% of the original mass of food sample.

1.1.6. The percentage of moisture is calculated as follows;

$$\% \text{ Moisture} = (w_2 - w_3) / (w_2 - w_1) \times 100$$

where : w_1 = initial weight of empty crucible

w_2 = weight of crucible + sample before drying

w_3 = final weight of crucible + sample after drying

1.2. Determination of crude protein by Macro Kjeldahl method (Osborne and Voogt,1972 ; AOAC,1990 ; James, 1995)

1.2.1. Accurately weigh 0.8 - 1.2 g of sample on the ashless filter paper and transfer to Kjeldahl digestion tube.

1.2.2. Add two Kjeltabs (Tecator,Sweden) to the tube, followed by 25 ml of concentrated sulfuric acid nitrogen-free, A.R. grade (E. Merk, Darmstadt, Germany) from an automatic dispenser.

1.2.3. Place the tube in the digestor (Buchi 430 Digestor) under the fume hood.

1.2.4. Heat at a mean temperature of 130°C for 30 min.

1.2.5. Increase the temperature over a 30 min period until the digest is boiling (about 350°C).

1.2.6. When the digest is a clear solution, continue boiling for a further 30 min.

1.2.7. Remove the tube from the digester and cool to room temperature.

1.2.8. Place the tube in the distillation unit (Buchi 322 distillation unit).

1.2.9. Place a conical flask containing 25 ml of 4% boric acid A.R. grade, E.Merk (containing modified methyl red indicator, 0.125 g methyl red and 0.0825 g methylene blue in 90% ethanol 100 ml) under the condenser outlet.

1.2.10. Dispense 100 ml of nitrogen-free distilled water and 120 ml of 40% sodium hydroxide (E. Merk, Darmstadt, Germany) , distill for 8 min.

1.2.11. Titrate the ammonium borate solution formed with 0.1 N sulfuric acid to a purplish-gray end point.

1.2.12. Carry out a blank determination using the same method but without sample to be tested.

1.2.13. Calculate the nitrogen content and hence the protein content of the sample given that:

$$\% \text{ Nitrogen content} = (v_2 - v_1) / w \times N \times 1.4$$

where : v_1 = volume (ml) of sulfuric acid solution required for the blank test

v_2 = volume (ml) of sulfuric acid solution required for the sample

w = weight (g) of the sample

N = normality of sulfuric acid

and : % Crude protein = % nitrogen x F

where : F = conversion factor (for soya = 5.71)

1.3. Determination of crude fat by the Soxhlet method (Osborne and Voogt, 1972; James, 1995)

1.3.1. Set up a Soxhlet extractor with reflux condenser and a distillation flask which has been previous dried and weighed.

1.3.2. Dry the sample according to method in 1.1.

1.3.3. Weigh accurately 2 - 3 g of dried sample into a fat-free extraction thimble, plug lightly with cotton wool.

1.3.4. Place the thimble in the extractor and connect a weighed flask containing 120 ml petroleum ether (Mallinckrodt Chemical, USA). Connect the extraction to a reflux condenser.

1.3.5. Extract the sample, under reflux, on Electromantle EMO 500/C (Electrothermal, England) for 12 h.

1.3.6. Evaporate the petroleum ether extract to dryness.

1.3.7. Dry the flask containing the fat residue in an hot-air oven at 100°C for 1 h, cool in a desiccator, and weigh.

1.3.8. Crude fat is calculated as a percentage of the sample taken given that :

$$\% \text{ Crude fat} = (w_2 - w_1) / w_3 \times 100$$

where : w_1 = weight of empty flask (g)

w_2 = weight of flask + fat (g)

w_3 = weight of sample taken (g)

1.4. Determination of ash by dry ashing method (Osborne and Voogt, 1972; James,1995)

1.4.1. Weigh accurately 4 - 6 g of the sample into a previously ignited, cooled and weighed crucible.

1.4.2. If the sample is liquid, pre-dry on a steam bath to prevent splitting during the charring stage.

1.4.3. Place the crucible on a hot plate under a fume hood and slowly increase the temperature until smoking ceases and the sample becomes thorough charred.

1.4.4. Transfer the crucible to a muffle furnace at about 550°C and leave until a white or light gray ash results. If the residue is black in color, moisten with a small amount of water, to dissolve salts, dry in an hot-air oven and repeat the ashing process.

1.4.5. Cool in a desiccator and reweigh.

1.4.6. Calculate the total ash as a percentage of original sample given that :

$$\% \text{ Ash} = (w_3 - w_1) / (w_2 - w_1) \times 100$$

where : w_1 = weight of empty crucible

w_2 = weight of crucible + sample before drying and/or ashing

w_3 = weight of crucible + ash

1.5. Determination of crude fiber by extraction unit for determining raw fiber content (Fiwe, VELP Scientific) (Osborne and Voogt, 1972; VELP Scientifica,1994).

1.5.1. Weigh accurately 1-2 g of sample into a crucible (glass filter, Robu-glass, Germany).

1.5.2. Add 1.25% sulfuric acid up to 150 ml notch, after preheating by the hot plate in order to reduce the time required for boiling.

1.5.3. Boil 30 min exactly from the onset of boiling.

1.5.4. Connect to vacuum for draining sulfuric acid.

1.5.5. Wash three times with 30 ml (crucible filled up to the top) of hot deionized water, connecting each time to compressed air for stirring the content of crucible.

1.5.6. After draining the last wash, add 150 ml of preheated sodium hydroxide (NaOH) 1.25%.

1.5.7. Boil 30 min.

1.5.8. Filter and wash as point 1.5.5.

1.5.9. Perform a last washing with cold deionized water aimed to cool the crucibles and then wash the crucible content three times with 25 ml of 95% ethyl alcohol, stirring each time by compressed air.

1.5.10. Remove the crucible and determine the dry weight after drying in an oven at 105°C for an hour or up to constant weight. Let cool in a desiccator. This weight represents the crude fiber plus ash content in comparison to initial weight.

1.5.11. When ash content is also required, the crucible are placed in a muffle at 500°C for 3 h and reweighed after cooling in a desiccator. The difference in weight in comparison to point 1.5.10 represents the crude fiber content without ash.

1.5.12. Calculate the crude fiber content as a percentage of sample given that:

$$\% \text{ crude fiber} = (w_2 - w_3) / w_1 \times 100$$

where : w_1 = weight of sample in the crucible (g)

w_2 = weight of the residue in the crucible (g)

w_3 = weight of ash in the crucible (g)

1.6. Determination of carbohydrates (James,1995)

Carbohydrates content is calculated by difference method, estimating all the other fractions by proximate analysis, i.e.

% Carbohydrates = 100 - (%moisture + %crude protein + %crude fat + % ash + %crude fiber)

2. Analysis of amino acid composition

Amino acid composition is determined by amino acid analyzer, Beckman system 6300 series (Osborne and Voogt, 1972; Matheson,1974; Meason et al., 1980)

2.1. Analysis of acid stable amino acids (except cystine and tryptophan)

2.1.1. Weigh accurately the sample containing 5-10 mg protein into hydrolysate tube.

2.1.2. Add 6 N hydrochloric acid (hydrochloric acid 1 ml per 1.5 mg protein) for hydrolyzing protein, then freeze.

2.1.3. Vacuum and stopper the tube.

2.1.4. Place the tube on the heating block, heat at 110°C for 24 h.

2.1.5. Cool and dry at 40°C by rotary vacuum evaporator.

2.1.6. Dissolve the residue in 20 ml of pH 2 sodium citrate buffer.

2.1.7. Separate the residue by centrifugation at 15,000 rpm for 5 min.

2.1.8. Transfer the supernatant solution into sample coil, place in autoinjection part of amino acid analyzer.

2.2. Analysis of cystine

Cystine is labile under acid hydrolysis condition, and require separate methods of analysis. It is therefore first oxidized with performic acid, to convert into cysteic acid and then freed from the protein by hydrolysis with hydrochloric acid.

2.2.1. Weigh accurately 20-30 mg (containing 10 mg of nitrogen content) into the tube.

2.2.2. Add performic acid (mix formic acid and hydrogen peroxide together to the ratio of formic acid to hydrogen peroxide 9:1, allow to stand for 1 h, then cool to 0°C).

2.2.3. Mix the sample with performic acid (point 2.2.2) thoroughly, stopper and leave to stand at 0°C for 16 h.

2.2.4. Follow the steps in 2.1.2 to 2.1.8.

2.3. Analysis of tryptophan

Tryptophan is determined by measuring the atomic absorption of the sample after hydrolysis of protein with barium hydroxide and dimethylamino benzaldehyde, according to the method of Matheson (1974).

2.4. Chemicals and working condition

2.4.1. Chemicals

- Sodium column 12 cm (Beckman No. 338052)
- Buffer pH 2.0, 3.0, 4.0, and 6.0
- Ninhydrin reagent
- 0.1 N Sodium hydroxide

2.4.2. Working condition for amino acid analyzer

- Temperature of column : 48, 75, and 77°C
- Buffer flow rate : 14 ml/h
- Ninhydrin flow rate : 7 ml/h
- Detector detect at 570 nm

3. Vitamin analysis

3.1. Vitamin B_1 (thiamin) (Kaew Kangsadalampai and Pongtorn Sungpuag, 1984)

Inoculum and standard :

Microorganism : *Lactobacillus fermini* ATCC 9338

Assay medium : add 0.7% D-xylose to Bacto-Thiamin Assay medium (Difco)

Preparation of standards :

1. Weigh 50 mg of thiamin into 500 ml volumetric flask and dilute to mark (solution a).

2. Pipette 5 ml of "solution a" into 500 ml volumetric flask and dilute to mark (solution b).

Standard a : Pipette 5 ml of "solution b" into 250 ml volumetric flask and dilute to mark (conc. 8 ng/ml).

Standard b : Pipette 2 ml of "solution b" into 100 ml volumetric flask and dilute to mark (conc. 20 ng/ml).

3. Pipette into 8 test tubes (in triplicate) according to the following:

conc.thiamin (ng/tube)	medium (ml)	dist. water (ml)	standard (ml)
0	2	2	0
4	2	0.5	1.5a
8	2	1	1.0a
12	2	0.5	1.5a
12	2	0	2a
20	2	1	1b
30	2	0.5	1.5b
40	2	0	2b

Procedure

1. weigh x g of sample into a flask.
2. Add 80 ml of distilled water and add 3 ml of 3N sulfuric acid.
3. Place on waterbath (100°C) for 30 min.
4. Cool, adjust pH to 4.5 with 3M sodium acetate.
5. Add 100 ml of Takadiastase (fungal enzyme) and keep overnight in incubator at 37°C.
6. Place into autoclave (120°C) for 5 min.
7. Cool and dilute according to the weight of sample.
8. Filter, adjust the filtrate to pH 6.1 with sodium hydroxide.
9. Dilute to concentration about 5 ng/ml.
10. Pipette in duplicate according to the following :

sample (ml)	dist.water (ml)	medium (ml)
0.5	1.5	2.0
1.0	1.0	2.0
2.0	0	2.0

11. Sterilize in autoclave at 100 °C for 15 min.
12. Cool, add one drop of inoculum.
13. Incubate at 37°C for 18 h.
14. Read in a spectrophotometer at 620 nm.

Calculation

Draw a standard curve by plotting the concentration of the standard on the x axis vs its corresponding absorbance (OD) on the y axis and determine the slope. Then calculate the thiamin concentration in the sample as follows :

$$\text{Thiamin (mg/100g)} = \frac{\text{OD sample x dilution}}{\text{slope x sample weight (g)}} \times 100$$

3.2. Vitamin B_2 (riboflavin) (Kaew Kangsadalampai and Pongtorn Sungpuag, 1984)

Inoculum and standard :

Microorganism : *Lactobacillus casei* ATCC 7469

Assay medium : Bacto - Riboflavin Assay medium (Difco)

Preparation of standards

1. Weigh 25 g of riboflavin into a beaker.
2. Add small amount of 0.02 N acetic acid.
3. Heat on waterbath until dissolved, dilute to 500 ml (solution a).
4. Pipette 10 ml of "solution a" into 500 ml volumetric flask and dilute to mark (solution b).

standard a : Pipette 2 ml of "solution b" into 100 ml volumetric flask and dilute to mark (conc. = 20 ng/ml).

standard b : Pipette 5 ml of "solution b" into 100 ml volumetric flask and dilute to mark (conc. 50 ng/ml).

5. Pipette into 8 test tubes (in triplicate) according to the following:

conc. riboflavin (ng/tube)	medium (ml)	dist. water (ml)	standard (ml)
0	2	2	0
10	2	1.5	0.5a
20	2	1	1a
30	2	0.5	1.5a
40	2	0	2a
50	2	1	1b
75	2	0.5	1.5b
100	2	0	2b

Procedure

1. Weigh x g of sample into a flask.
2. Add 100 ml 0.1 N hydrochloric acid.
3. Place in autoclave (120°C) for 15 min.
4. Cool, adjust pH to 4.5 with 3 M sodium acetate.
5. Dilute according to the weight of sample.
6. Filter, adjust filtrate to pH 6.6 with sodium hydroxide.
7. Dilute to concentration about 20 ng/ml.
8. Pipette in duplicate according to the following :

sample (ml)	dist. water (ml)	medium (ml)
0.5	1.5	2.0
1.0	1.0	2.0
2.0	0	2.0

9. Sterilize in autoclave (120°C) for 10 min.
10. Cool, and add one drop of inoculum
11. Incubate at 37°C overnight
12. Read in spectrophotometer at 620 nm.

calculation

Draw a standard curve by plotting the concentration of the standard on the x axis vs its corresponding absorbance (OD) on the y axis and determine the slope. Then calculate the riboflavin concentration in the sample as follows ;

$$\text{Riboflavin (mg/100g)} = \frac{\text{OD sample x dilution}}{\text{slope x sample weight (g)}} \times 100$$

3.3. Niacin (Kaew Kangsadalampai and Pongtorn Sungpuag, 1984)

Inoculum and standard :

Microorganism : *Lactobacillus plantarum* ATCC 8014

Assay medium : Bacto-Niacin Assay Medium (Difco)

Preparation of standards

1. Weigh 50 mg of nicotinic acid into a beaker.
2. Dissolve with a little water and dilute 500 ml with 500 volumetric flask (solution a).
3. Pipette 1 ml of "solution a" into 100 ml volumetric flask and dilute to mark (solution b).

Standard a : Pipette 2 ml of solution b into 100 ml volumetric flask and dilute to mark (20 ng/ml).

Standard b : Pipette 5 ml of solution b into 100 ml volumetric flask and dilute to mark (50 ng/ml).

4. Pipette into 8 test tubes in triplicate according to the following :

conc. niacin (ng/ml)	medium (ml)	dist. water (ml)	standard (ml)
0	2	2	0
10	2	1.5	0.5a
20	2	1	1.0a
30	2	0.5	1.5a
40	2	0	2.0a
50	2	1	1.0b
75	2	0.5	1.5b
100	2	0	2.0b

Procedure

1. Weigh x g of sample into a flask.
2. Add 80 ml of 1 N hydrochloric acid.
3. Place in autoclave (120°C) for 20 min.
4. Cool, adjust pH to 6.8 with sodium hydroxide.
5. Dilute according to the weight of sample.
6. Filter, dilute clear filtrate to a concentration of about 20 ng/ml.
7. Pipette in duplicate according to the following :

sample (ml)	dist. water (ml)	medium (ml)
0.5	1.5	2
1	1	2
2	0	2

8. Sterilize in an autoclave (120°C) for 10 min.
9. Cool, add one drop of inoculum and mix thoroughly.
10. Incubate at 37°C for 18 h.
11. Read in a spectrophotometer at 620 nm.

Calculation

Draw a standard curve by plotting the concentration of the standard on the x axis vs its corresponding absorbance (OD) on the y axis and determine the slope. Then calculate the niacin concentration in the sample as follows ;

$$\text{Niacin (mg/100g)} = \frac{\text{OD sample x dilution x 100}}{\text{slope x sample weight (g)}}$$

3.4. Vitamin B_6 (pyridoxine) (Osborne and Voogt,1978)

Procedure

Extraction

1. Weigh between 0.5 g and 15 g into a 400 ml beaker, containing not more than 12.5 µg vitamin B_6.
2. Add 180 ml of 0.44 N sulfuric acid.
3. Cover the beaker with an aluminium foil cap.
4. Autoclave for 5 min at 121°C.
5. Remove from autoclave and cool in waterbath.
6. Add 20 ml of 4 N sodium hydroxide, using a measuring cylinder.
7. Adjust to pH 4.5 on a pH meter, using 1 N and 0.1 N sodium hydroxide solution.
8. Rinse electrode with water.
9. Transfer contents of beaker to volumetric flask and dilute to 250 ml with water.
10. Filter through Whatman No. 42 paper (or No. 541 paper if samples contain no fat).
11. Store the filtrate in the refrigerator until needed (extracts may be stored for up to 36 h).
12. Dilute the filtrate if necessary to given pyridoxine concentration of approximately 50 ng/ml.

Reagent blanks are taken through the same procedure in duplicate.

Praparation of assay solution

1. Serially dilute the pyridoxine stock standard solution 5 ml to 500 ml and 5 ml to 100 ml to given a pyridoxine working solution (50 ng/ml). Pipette 0, 0.04, 0.08, 0.12, 0.16, and 0.2 ml of working standard solution in duplicate into assay tubes.
2. Pipette 0.05, 0.10, and 0.20 ml aliquots of each sample extraction in duplicate into assay tubes.

3. Using a suitable dispenser add 5 ml of double strength medium to each assay tube.

4. Dipense 5 ml aliquots of medium into a further set of assay tubes to act as blanks.

5. Cap each tube with polypropylene cap.

6. Autoclave the assay for 5 min at 121°C.

7. Remove from autoclave and cool to below 30°C in waterbath.

Preparation of the inoculum

1. Transfer cells of *Saccharomyces carlsbergensis* from the agar slope to a tube containing 5 ml of sterile double strength pyridoxine Y medium.

2. Incubate for 20 h at a temperature between 28 and 30°C with the loosely capped tube inclined at an angle of 45°C.

3. Prepare inoculum by diluting the subculture aseptically with sterile double strength medium until a reading of 50 is obtained on the standard perpex scale.

Inoculation and incubation of tubes

1. Fill a sterile 10 ml pipette with the inoculum.

2. Remove the cap from the first assay tube.

3. Immediately add 1 drop only of the inoculum to the tube and replace the cap loosely.

4. Repeat the process for each tube in the assay except the uninoculated blank tubes.

5. Place the assay in an incubator with each tube in an inclined position.

6. Incubate at 30°C for 2 to 22 h.

7. Remove tubes from the incubator and add 7 ml of water to each.

Measurement of growth

1. Mix the contents of each assay tube on a Vortex mixer immediately before reading the nephelometer.

2. Place an uninoculated blank tube in the instrument and adjust the reading to zero.

3. Place an assay tube containing 0.2 ml of standard in the instrument and set the reading to approximately 95-100% full scale deflection.

4. Read the remaining assay solution, mixing each before placing in the instrument and record the galvanometer readings.

Calculation

1. Draw a standard curve by plotting nephelometer reading against nanogram pyridoxine per tube for standards.

2. From the graph obtain the amount of pyridoxine (ng) in each pair of assay tubes, x_1, x_2, x_3.

Let : Aliquots (ml) taken for the assay be v_1, v_2, v_3 respectively.

Then : Concentration of pyridoxine in sample extract =

$(x_1/v_1 + x_2/v_2 + x_3/v_3)/3$ ng/ml = a ng/ml.

Let : Weight (g) of sample = w

Volume (ml) of extract = v

Then : Pyridoxine content (mg/100 g) = $(a \times v \times 10^2) / (w \times 10^6)$

= $(a \times v) / (w \times 10^4)$

3.5. Vitamin B_{12} (cobalamine) (Osborne and Voogt,1978)

Procedure

Extraction

1. Weigh out between 0.25 g and 5.0 g of sample into 25 mm x 150 mm assay tubes containing not more than 40 ng B_{12}.

2. Add 20 ml of acetate buffer solution and 2 drops of sodium cyanide solution.

3. Cover the tubes with aluminium foil caps,

4. Immerse in a boiling water bath for 30 min.

5. Cool and dilute the extracts to 50 ml with water.

6. Filter through Whatman No. 42 paper (or No. 541 paper if samples do not contain fat).

7. If necessary the filtrate can be stored in refrigerator overnight.

Preparatiopn of inoculum

1. Subculture the organism overnight from the *Lactobacilus leichmanii* culture and transfer to a tube containing 5 ml sterile micro inoculum broth.

2. Incubate overnight at 37°C .

3. Mix and transfer 1 drop aseptically to a tube containing 5 ml of single strength vitamin B_{12} medium fortified with vitamin B_{12}.

4. Incubate for 6 h at 37°C.

5. Prepare the inoculum by transferring 1 drop with a sterile pipette to an assay tube containing 10 ml of single strength assay media.

Preparation of assay solutions

1. From the stock solution A, dilute 2 ml + 2 drops of sodium cyanide to 1000 ml with water to give standard B (20 ng/ml).

2. From standard B dilute 4 ml to 100 ml to give standard C (0.8 ng/ml).

3. Pipette 0, 0.05, 0.1, 0.15, 0.2, and 0.25 ml aliquots of standard solution C in duplicate into assay tubes.

4. Dilute sample extracts, if necessary, to give a concentration of approximately 0.80 ng/ml.

5. Pipette 0.05, 0.1, and 0.2 ml aliquots of each extract in duplicate into assay tubes.

6. Using a suitable dispenser, add 10 ml of single strength media to each assay tube.

7. Add 10 ml of media into a further 5 assay tubes to act as blanks or to use in the preparation of inoculum.

8. Cap all the tubes.

9. Autoclave the whole assay for 5 min at 121°C (15 lb/in^2).

10. Remove from autoclave and cool to below 30°C in a waterbath.

Inoculation and incubation of tubes

1. Loosen all caps, but leave tubes covered.
2. Fill a sterile 10 ml pipette with the inoculum.
3. Remove cap from first assay tube, then add 1 drop only of inoculum and rapidly replace the cap.
4. Repeat the process for each tube in the assay except the 5 blanks (uninoculated blanks).
5. Place the assay in the incubator for 22 h at 37°C.

Measurement of growth

1. Mix contents of each set of tubes using a Vortex mixer, just before reading. Read the assay an EEL nephelometer.
2. Place an inoculated blank tube in nephelometer and adjust the reading to zero.
3. Place an assay tube containing the top level of standard in the instrument and set the readings to approximately 95-100% full scale deflection.
4. Repeat the previous two procedure until the instrument is stable at 0 and a point between 95-100% full scale deflection.
5. Read the remaining assay tubes and record the galvanometer readings.

calculation

1. Draw a standard curve by plotting nephelometer readings against picogram vitamin B_{12} per tube for standards.
2. From the graph obtain the amount of vitamin B_{12} (pg) in each pair of assay tubes x_1, x_2, x_3.

Let : Aliquots (ml) taken for the assay be v_1, v_2, v_3 respectively.

Then : Concentration of vitamin B_{12} in sample extract

$$(x_1/v_1 + x_2/v_2 + x_3/v_3)/3 = a \text{ pg/ml.}$$

Let : Weight (g) of sample = w

Volume (ml) of extract = v

Then : Vitamin B_{12} content (μg/100 g) = $(a \times v \times 10^2) / (w \times 10^6)$

= $(a \times v) / (w \times 10^4)$

3.6. Pantothenic acid (Murata et al.,1967)

Samples are homogenized with water, then heated for 30 min in boiling waterbath. The extracts are treated with intestinal alkaline phosphatase and pigeon liver enzyme in pH 8.3 Tris buffer for 4 h at 37°C (Novelli et al.,1951) , heated in an autoclave, cooled, adjusted to pH 5.0, and centrifuged. The assay is run on the supernatant, according to the method of Hoag et al. (1945), using *Lactobacillus arabinosus* ATCC 8014, as test organism. The tubes are incubated for 15 h at 37°C, and the turbidity is read in a photoelectric colorimeter using 620 mμ filter.

Table A.1 : Standard protein of FAO/WHO 1973
(Joint FAO/WHO Ad Hoc Expert Committee 1973)

Amino acid	mg/g protein
Isoleucine	40
Leucine	70
Lysine	55
Methionine + Cystine	35
Threonine	40
Phenylalanine + Tyrosine	60
Valine	50
Tryptophan	10

APPENDIX II

TECHNIQUES IN MICROBIOLOGY

Microbiological culture media

1. Potato dextrose agar (PDA)

Potato, peeled and cut up	200.0	g
Dextrose	10.0	g
Agar	15.0	g
Distilled water	1.0	l

2. Nutrient agar (NA)

Nutrient agar	31.0	g
Distilled water	1.0	l

Microorganisms

1. ***Rhizopus oligosporus*** TISTR 3001 was obtained from Microbiological Resources Center (Bangkok MIRCEN) , Thailand Institute of Scientific and Technological Research (TISTR). Stock of strain was maintained on PDA slopes at 5°C.

2. ***Bacillus subtilis*** TISTR 1 was furnished from Microbiological Resources Center (Bangkok MIRCEN) , Thailand Institute of Scientific and Technological Research (TISTR). Stock of strain was maintained on NA slopes at 5°C.

Subculturing

At bimonthly intervals, a subculture was made of a stock strain kept. This was done by using a sterile loop to pick up a few cells of the microorganisms and then making a streak of these on an agar slope in a test tube.

The determination of microbial numbers

1. Total spore count of *Rhizopus oligosporus* TISTR 3001 (Gerhardt,1981; วัฒนาลัย ปานบ้านเกร็ด และ สรวง อุดมวรภัณฑ์, 2536)

A spore suspension of *Rhizopus oligosporus* TISTR 3001 on PDA slant was prepared by introducing 5 ml sterile 0.02% tween 80 in distilled water onto 3-day-old culture incubated at 30°C. Dilute the suspension so that 1 ml would be expected to yield 1 x 10^7 spores. The number of spores in the suspension was determined by haemocytometer.

Procedure

1. Clean the chamber and the cover slip and let it air dry.
2. Make preliminary estimation of the concentration of spores. Proceed to the next step if the concentration is less than 3 x 10^8 spores per ml. Otherwise, make a primary dilution of the spore suspension in 0.02% Tween 80 in distilled water.
3. Immediately fill the chamber with spore suspension. Let the chamber rest for 1-2 min.
4. Examine with microscope under a high dry objective lens.
5. Count the spores lying within small squares. Optically, the number in each small square should be in the rang of 5 to 15. Score the spores that cross the bounderies of a square if they are on the upper or left side but not if they are on the lower or right side. It is best to count squares chosen in a systematic fashion, such as the four corner squares and the major diagonal squares. This prevents counting the same square twice and average a possible geometric gradient of spores in the chamber.

6. Calculate the number of spores by the following formula :

Small square counted area	= 1/5 x 1/5 x 1/10 x 5	mm^3
(A,B,C,D,E)	= 1/50	mm^3
Number of spores in 1/50 mm^3	= X	spores
Total initial spores counted	= 5X x 10^4 x dilution factor	spores/ml

Figure 5 : The counting chamber of haemocytometer showing square chosen (A,B,C,D,E)

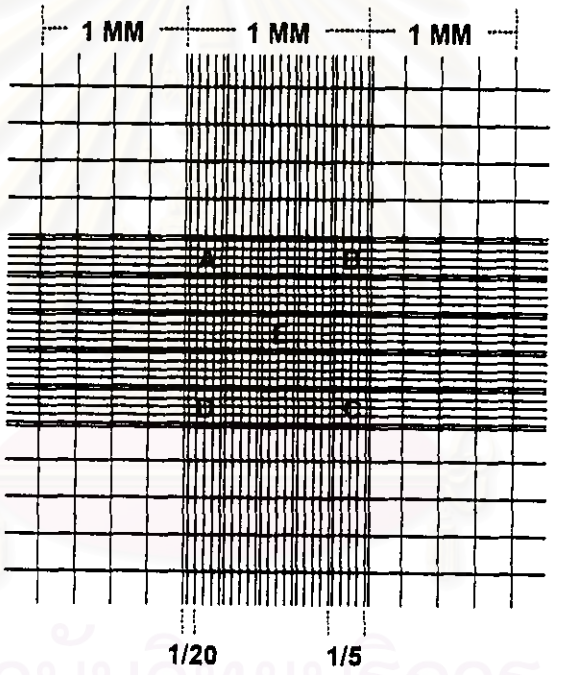
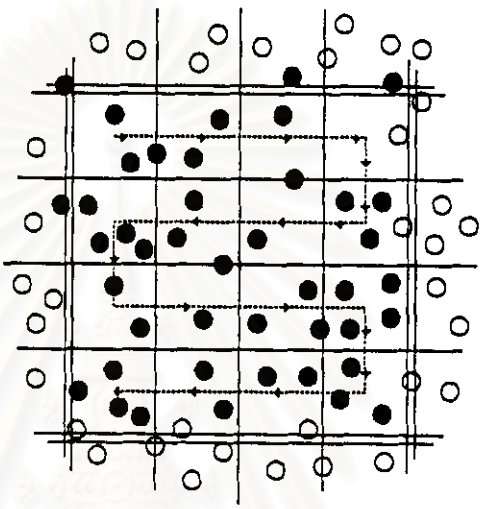


Figure 6 : Method of spore counting

● score

° unscore



2. Total cells count of *Bacillus subtilis* TISTR 1 (Seeley and Demark, 1972; Poolsri Suwisuttagul, 1979)

2.1. Pour plate method (Seeley and Demark, 1972; Poolsri Suwisuttagul, 1979)

Bacillus subtilis TISTR 1 was grown on nutrient agar at 30°C for 2 days. The culture was suspended in sterile diluent (0.85% sodium chloride in distilled water).

Procedure

To dilute the culture suspension, 1-ml portion was diluted stepwise through a series of tubes containing 9 ml of 0.85% sodium chloride in distilled water. By containing this dilution stepwise through additional dilution tubes, we could dilute the culture suspension at 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and higher dilutions.

Pipette 1 ml of each dilution into each of two sterile petri dishes. Promptly pour the melted, cooled nutrient agar into the petri dishes and mix the culture suspension thoroughly with the agar by tilting or rotating the dishes. Allow the contents to solidify at room temperature and then incubate at 30°C for 24 h in an inverted position. Following incubation, the number of colonies developing on each dish in the range of 30 to 300 were counted. The number of bacteria of the initial culture suspension was then determined by multifying the number of colonies by the degree of dilution (dilution factor) of the dish that was being counted and express the average for the two dishes in terms of the number of cellls per ml of the initial culture suspension.

2.2. Turbidimetric estimation of cell growth (Seeley and Demark, 1972)

In the use of turbidimetry to measure cell growth, the turbidity of the bacterial culture is correlated with some other known measure of cell growth, such as the total number of bacteria as determined by the plate method. In this

experiment, we determined the turbidity of several dilutions of a 24-hour culture of *Bacillus subtilis* TISTR1 by spectronic 21 (Milton Roy Company) at 600 nm and also made plate counts on the culture to determine the cell number. From the results, we could find the correlation between turbidity and cell count and we would be able to estimate the number of cells present in the suspension which be used in inoculation step of tooa-nao production.

APPENDIX III

STATISTICAL ANALYSIS

1. Mean ($\bar{x}$)

$$\bar{x} = \frac{\sum x_i}{n}$$

where n = sample size

2. Standard Deviation (S.D.)

$$\text{S.D.} = \sqrt{\frac{\sum(x_i - \bar{x})^2}{n-1}}$$

3. One - way analysis of variance - ANOVA (Anderson, Sweeney, and Williams, 1981; Garcia - Diaz and Phillips, 1995)

Basic notation and data format

	Treatments					
	j = 1	j = 2	j = 3...	j	...j = k-1	j = 1
$i = 1$	y_{11}	y_{12}	y_{13}	y_{1j}	$y_{1,k-}$	y_{1k}
$i = 2$	y_{21}	y_{22}	y_{23}	y_{2j}	$y_{2,k-1}$	y_{2k}
⋮	⋮	⋮	⋮	⋮	⋮	⋮
$i = n_i$	$y_{n1,1}$	$y_{n2,2}$	$y_{n3,3}$	$y_{nj,j}$	$y_{nk-1,k-1}$	$y_{nk,k}$
total	$T_{.1}$	$T_{.2}$	$T_{.3}$	$T_{.j}$	$T_{.k-1}$	$T_{.1}$
sample size	n_1	n_2	n_3	n_j	n_{k-1}	n_k
sample mean	$\bar{y}_{.1}$	$\bar{y}_{.2}$	$\bar{y}_{.3}$	$\bar{y}_{.j}$	$\bar{y}_{.k-1}$	$\bar{y}_{.k}$

The general analysis of variance table

Source of variation	df	SS	MS	F
Between treatments	$k-1$	$SS_{treatment}$	$\frac{SS_{treatment}}{(k-1)}$	$\frac{MS_{treatment}}{MS_{error}}$
Within treatment	$N-k$	SS_{error}	$\frac{SS_{error}}{(N-k)}$	
Total variation	$N-1$	SS_{total}		

$$SS_{treatment} = \sum_{j} \frac{T_j^2}{n_j} - \frac{T_.^2}{N}$$

$$SS_{total} = \sum_{i}\sum_{j} y_{ij}^2 - \frac{T_.^2}{N}$$

$$SS_{error} = SS_{total} - SS_{treatment}$$

where i = number of sample in each treatment 1, 2, ..., n_j

j = number of treatment 1, 2, ..., k

$T_.$ = the grand total $\sum_{j=1}^{k} T_{.j}$

N = the total number of observations $\sum_{j=1}^{k} n_j$

4. Duncan's multiple range test

The Least Significant Difference (LSD) procedure is only use when the basic ANOVA test shown that at least one treatment mean is significantly different from the others i.e. when the treatment source of variation is formed to be significant by the F-Statistic.

It is easy to apply the LSD procedure, since one simply compares the observed absolute value of the difference between each pair of means. All pairs of treatment means whose difference exceeds the critical value are considered as significant.

$$R_C = \left[\frac{MS_{error}}{n}\right]^{1/2} Q'_{(r,\alpha,f)}$$

where

R_C = Least Significant Difference

Q = Values of Duncan multiple range test

r = The number of steps between $\overline{Y}_j$ and $\overline{Y}_i$,that is

$$r = j - i + 1$$

α = The significance level of the test

f = Degree of freedom within treatments (N-k)

n = Number of sample in each treatment

Example

Treatment				
A	B	C	D	E
1.25	2.25	6.75	3.25	5.50
1.50	4.50	4.25	3.50	3.50
2.50	4.75	6.25	4.75	7.25
1.30	3.25	7.50	3.50	4.75
2.70	1.50	4.50	5.50	4.00
1.20	3.75	4.75	3.75	6.25
1.80	4.50	8.00	7.25	3.75

	A ($j=1$)	B ($j=2$)	C ($j=3$)	D ($j=4$)	E ($j=5$)	Total
T_j	12.25	24.50	31.50	35.00	42.00	T = 145.25
n_j	7	7	7	7	7	N = 35
$\overline{Y}_j$	1.75	3.50	4.50	5.00	6.00	

Results from Duncan's test

	$Y_{(1)}$	$Y_{(2)}$	$Y_{(3)}$	$Y_{(4)}$	$Y_{(5)}$
$Y_{(1)}$	-	1.75*	2.75*	3.25*	4.25*
$Y_{(2)}$	-	-	1.00	1.50*	2.50*
$Y_{(3)}$	-	-	-	0.50	1.50*
$Y_{(4)}$	-	-	-	-	1.00
$Y_{(5)}$	-	-	-	-	-

* indicate a significant difference between means.

The critical value for R_C in this case are determined using

$$R_C = \left[\frac{MS_{error}}{n}\right]^{1/2} Q'_{(r,0.05,30)}$$

where the required critical value of the Q-Statistic come from values for Duncan's multiple range test, $\alpha = 0.05$
The following critical value are obtained :

$r = 2 : Q'_{(2,0.05,30)} = 2.89 \quad R_C = 1.41$
$r = 3 : Q'_{(3,0.05,30)} = 3.04 \quad R_C = 1.48$
$r = 4 : Q'_{(4,0.05,30)} = 3.13 \quad R_C = 1.52$
$r = 5 : Q'_{(5,0.05,30)} = 3.20 \quad R_C = 1.56$

Table A.2 : Analysis of variance table of moisture content (dry weight basis)

Source of variation	df	SS	MS	F	
				calculated	α=0.05
Between treatments	5	20.320	4.0640	72.57*	3.11
Within treatment	12	0.673	0.0560		
Total variation	17	20.993			

* At least one treatment mean is significantly different from the others.

Results from Duncan's test

	$Y_{(1)}$	$Y_{(2)}$	$Y_{(3)}$	$Y_{(4)}$	$Y_{(5)}$	$Y_{(6)}$
$Y_{(1)}$	-	0.20	1.31*	1.44*	1.70*	3.23*
$Y_{(2)}$	-	-	1.11*	1.24*	1.50*	3.03*
$Y_{(3)}$	-	-	-	0.13	0.39	1.92*
$Y_{(4)}$	-	-	-	-	0.26	1.79*
$Y_{(5)}$	-	-	-	-	-	1.53*
$Y_{(6)}$	-	-	-	-	-	-

* indicate a significant difference between means.

The critical value for R_C are as follows;

$$R_C = \left[\frac{MS_{error}}{n}\right]^{1/2} Q'_{(r,0.05,12)}$$

$r = 2 : Q'_{(2,0.05,12)} = 3.08 \quad R_C = 0.4207$
$r = 3 : Q'_{(3,0.05,12)} = 3.23 \quad R_C = 0.4412$
$r = 4 : Q'_{(4,0.05,12)} = 3.33 \quad R_C = 0.4549$
$r = 5 : Q'_{(5,0.05,12)} = 3.36 \quad R_C = 0.4590$
$r = 6 : Q'_{(6,0.05,12)} = 3.40 \quad R_C = 0.4644$

$Y_{(1)}$ = Soybean residue tempeh : 3.97 ^{c}

$Y_{(2)}$ = Unfermented soybean : 4.17 ^{c}

$Y_{(3)}$ = Soybean tooa-nao : 5.28 ^{b}

$Y_{(4)}$ = Soybean tempeh : 5.41 ^{b}

$Y_{(5)}$ = Unfermented soybean residue : 5.67 ^{b}

$Y_{(6)}$ = Soybean residue tooa-nao : 7.20 ^{a}

Table A.3 : Analysis of variance table of crude protein content

Source of variation	df	SS	MS	F	
				calculated	α=0.05
Between treatments	5	834.210	166.8400	9533.71*	3.11
Within treatment	12	0.210	0.0175		
Total variation	17	834.420			

* At least one treatment mean is significantly different from the others.

Results from Duncan's test

	$Y_{(1)}$	$Y_{(2)}$	$Y_{(3)}$	$Y_{(4)}$	$Y_{(5)}$	$Y_{(6)}$
$Y_{(1)}$	-	0.42*	0.61*	13.37*	14.09*	14.36*
$Y_{(2)}$	-	-	0.19	12.95*	13.67*	13.94*
$Y_{(3)}$	-	-	-	12.76*	13.48*	13.75*
$Y_{(4)}$	-	-	-	-	0.72*	0.99*
$Y_{(5)}$	-	-	-	-	-	0.27*
$Y_{(6)}$	-	-	-	-	-	-

* indicate a significant difference between means.

The critical value for R_C are as follows;

$$R_C = \left[\frac{MS_{error}}{n}\right]^{1/2} Q'_{(r,0.05,12)}$$

r = 2 : $Q'_{(2,0.05,12)}$ = 3.08 R_C = 0.2340

r = 3 : $Q'_{(3,0.05,12)}$ = 3.23 R_C = 0.2450

r = 4 : $Q'_{(4,0.05,12)}$ = 3.33 R_C = 0.2530

r = 5 : $Q'_{(5,0.05,12)}$ = 3.36 R_C = 0.2550

r = 6 : $Q'_{(6,0.05,12)}$ = 3.40 R_C = 0.2580

$Y_{(1)}$ = Unfermented soybean residue : 25.45 [e]

$Y_{(2)}$ = Soybean residue tooa-nao : 25.87 [d]

$Y_{(3)}$ = Soybean residue tempeh : 26.06 [d]

$Y_{(4)}$ = Unfermented soybean : 38.82 [c]

$Y_{(5)}$ = Soybean tooa-nao : 39.54 [b]

$Y_{(6)}$ = Soybean tempeh : 39.81 [a]

Table A.4 : Analysis of variance table of crude fat content

Source of variation	df	SS	MS	F	
				calculated	α=0.05
Between treatments	5	449.570	89.9140	1729.12*	3.11
Within treatment	12	0.620	0.0520		
Total variation	17	450.190			

* At least one treatment mean is significantly different from the others.

Results from Duncan's test

	$Y_{(1)}$	$Y_{(2)}$	$Y_{(3)}$	$Y_{(4)}$	$Y_{(5)}$	$Y_{(6)}$
$Y_{(1)}$	-	0.21	1.74*	7.29*	9.10*	13.35*
$Y_{(2)}$	-	-	1.53*	7.08*	8.89*	13.14*
$Y_{(3)}$	-	-	-	5.55*	7.36*	11.61*
$Y_{(4)}$	-	-	-	-	1.81*	6.06*
$Y_{(5)}$	-	-	-	-	-	4.25*
$Y_{(6)}$	-	-	-	-	-	-

* indicate a significant difference between means.

The critical value for R_C are as follows;

$$R_C = \left[\frac{MS_{error}}{n}\right]^{1/2} Q'_{(r,0.05,12)}$$

r = 2 : $Q'_{(2,0.05,12)}$ = 3.08 R_C = 0.4070

r = 3 : $Q'_{(3,0.05,12)}$ = 3.23 R_C = 0.4260

r = 4 : $Q'_{(4,0.05,12)}$ = 3.33 R_C = 0.4400

r = 5 : $Q'_{(5,0.05,12)}$ = 3.36 R_C = 0.4440

r = 6 : $Q'_{(6,0.05,12)}$ = 3.40 R_C = 0.4490

$Y_{(1)}$ = Soybean residue tooa-nao : 13.23 ^{e}

$Y_{(2)}$ = Soybean residue tempeh : 13.44 ^{e}

$Y_{(3)}$ = Unfermented soybean residue : 14.97 ^{d}

$Y_{(4)}$ = Soybean tempeh : 20.52 ^{c}

$Y_{(5)}$ = Unfermented soybean : 22.33 ^{b}

$Y_{(6)}$ = Soybean tooa-nao : 26.58 ^{a}

Table A.5 : Analysis of variance table of ash content

Source of variation	df	SS	MS	F	
				calculated	α=0.05
Between treatments	5	5.387	1.0770	828.46*	3.11
Within treatment	12	0.015	0.0010		
Total variation	17	5.402			

* At least one treatment mean is significantly different from the others.

Results from Duncan's test

	$Y_{(1)}$	$Y_{(2)}$	$Y_{(3)}$	$Y_{(4)}$	$Y_{(5)}$	$Y_{(6)}$
$Y_{(1)}$	-	0	0.11*	1.02*	1.08*	1.24*
$Y_{(2)}$	-	-	0.11*	1.02*	1.08*	1.24*
$Y_{(3)}$	-	-	-	0.91*	0.97*	1.13*
$Y_{(4)}$	-	-	-	-	0.06	0.22*
$Y_{(5)}$	-	-	-	-	-	0.16*
$Y_{(6)}$	-	-	-	-	-	-

* indicate a significant difference between means.

The critical value for R_C are as follows;

$$R_C = \left[\frac{MS_{error}}{n}\right]^{1/2} Q'_{(r,0.05,12)}$$

$r = 2 : Q'_{(2,0.05,12)} = 3.08 \quad R_C = 0.0650$

$r = 3 : Q'_{(3,0.05,12)} = 3.23 \quad R_C = 0.0680$

$r = 4 : Q'_{(4,0.05,12)} = 3.33 \quad R_C = 0.0700$

$r = 5 : Q'_{(5,0.05,12)} = 3.36 \quad R_C = 0.0706$

$r = 6 : Q'_{(6,0.05,12)} = 3.40 \quad R_C = 0.0714$

$Y_{(1)}$	= Unfermented soybean residue	: 3.33 [d]
$Y_{(2)}$	= Soybean residue tooa-nao	: 3.33 [d]
$Y_{(3)}$	= Soybean residue tempeh	: 3.44 [c]
$Y_{(4)}$	= Unfermented soybean	: 4.35 [b]
$Y_{(5)}$	= Soybean tempeh	: 4.41 [b]
$Y_{(6)}$	= Soybean tooa-nao	: 4.57 [a]

Table A.6 : Analysis of variance table of crude fiber content

Source of variation	df	SS	MS	F	
				calculated	α=0.05
Between treatments	5	1008.644	201.7290	3955.471*	3.11
Within treatment	12	0.609	0.0510		
Total variation	17	1009.253			

* At least one treatment mean is significantly different from the others.

Results from Duncan's test

	$Y_{(1)}$	$Y_{(2)}$	$Y_{(3)}$	$Y_{(4)}$	$Y_{(5)}$	$Y_{(6)}$
$Y_{(1)}$	-	2.25*	2.95*	14.41*	17.44*	17.52*
$Y_{(2)}$	-	-	0.70*	12.16*	15.19*	15.27*
$Y_{(3)}$	-	-	-	11.46*	14.49*	14.57*
$Y_{(4)}$	-	-	-	-	3.03*	3.11*
$Y_{(5)}$	-	-	-	-	-	0.08
$Y_{(6)}$	-	-	-	-	-	-

* indicate a significant difference between means.

The critical value for R_C are as follows;

$$R_C = \left[\frac{MS_{error}}{n}\right]^{1/2} Q'_{(r,0.05,12)}$$

$r = 2 : Q'_{(2,0.05,12)} = 3.08 \quad R_C = 0.4000$
$r = 3 : Q'_{(3,0.05,12)} = 3.23 \quad R_C = 0.4200$
$r = 4 : Q'_{(4,0.05,12)} = 3.33 \quad R_C = 0.4330$
$r = 5 : Q'_{(5,0.05,12)} = 3.36 \quad R_C = 0.4370$
$r = 6 : Q'_{(6,0.05,12)} = 3.40 \quad R_C = 0.4420$

$Y_{(1)}$	= Soybean tempeh	: 5.49 [e]
$Y_{(2)}$	= Unfermented soybean	: 7.74 [d]
$Y_{(3)}$	= Soybean tooa-nao	: 8.44 [c]
$Y_{(4)}$	= Soybean residue tempeh	: 19.90 [b]
$Y_{(5)}$	= Unfermented soybean residue	: 22.93 [a]
$Y_{(6)}$	= Soybean residue tooa-nao	: 23.01 [a]

Table A.7 : Analysis of variance table of moisture content (fresh weight basis)

Source of variation	df	SS	MS	F	
				calculated	α=0.05
Between treatments	5	127.550	25.5100	398.59*	3.11
Within treatment	12	0.770	0.0640		
Total variation	17	128.320			

* At least one treatment mean is significantly different from the others.

Results from Duncan's test

	$Y_{(1)}$	$Y_{(2)}$	$Y_{(3)}$	$Y_{(4)}$	$Y_{(5)}$	$Y_{(6)}$
$Y_{(1)}$	-	0.12	0.25	4.49*	5.07*	6.41*
$Y_{(2)}$	-	-	0.13	4.37*	4.95*	6.29*
$Y_{(3)}$	-	-	-	4.24*	4.82*	6.16*
$Y_{(4)}$	-	-	-	-	0.58*	1.92*
$Y_{(5)}$	-	-	-	-	-	1.34*
$Y_{(6)}$	-	-	-	-	-	-

* indicate a significant difference between means.

The critical value for R_C are as follows;

$$R_C = \left[\frac{MS_{error}}{n}\right]^{1/2} Q'_{(r,0.05,12)}$$

$r = 2 : Q'_{(2,0.05,12)} = 3.08 \quad R_C = 0.4497$
$r = 3 : Q'_{(3,0.05,12)} = 3.23 \quad R_C = 0.4716$
$r = 4 : Q'_{(4,0.05,12)} = 3.33 \quad R_C = 0.4862$
$r = 5 : Q'_{(5,0.05,12)} = 3.36 \quad R_C = 0.4906$
$r = 6 : Q'_{(6,0.05,12)} = 3.40 \quad R_C = 0.4964$

$Y_{(1)}$ = Soybean residue tooa-nao : 51.61 [d]

$Y_{(2)}$ = Unfermented soybean residue : 51.73 [d]

$Y_{(3)}$ = Soybean residue tempeh : 51.86 [d]

$Y_{(4)}$ = Unfermented soybean : 56.10 [c]

$Y_{(5)}$ = Soybean tempeh : 56.68 [b]

$Y_{(6)}$ = Soybean tooa-nao : 58.02 [a]

Table A.8 : Analysis of variance table of protein digestibility

Source of variation	df	SS	MS	F	
				calculated	α=0.05
Between treatments	5	374.040	74.8080	6.087*	3.11
Within treatment	12	147.470	12.2890		
Total variation	17	521.510			

* At least one treatment mean is significantly different from the others.

Results from Duncan's test

	$Y_{(1)}$	$Y_{(2)}$	$Y_{(3)}$	$Y_{(4)}$	$Y_{(5)}$	$Y_{(6)}$
$Y_{(1)}$	-	5	7.27*	8.03*	8.53*	15.3*
$Y_{(2)}$	-	-	2.27	3.03	3.53	10.3*
$Y_{(3)}$	-	-	-	0.76	1.26	8.03*
$Y_{(4)}$	-	-	-	-	0.50	7.27*
$Y_{(5)}$	-	-	-	-	-	6.77*
$Y_{(6)}$	-	-	-	-	-	-

* indicate a significant difference between means.

The critical value for R_C are as follows;

$$R_C = \left[\frac{MS_{error}}{n}\right]^{1/2} Q'_{(r,0.05,12)}$$

r = 2 : $Q'_{(2,0.05,12)}$ = 3.08 R_C = 6.2340

r = 3 : $Q'_{(3,0.05,12)}$ = 3.23 R_C = 6.5380

r = 4 : $Q'_{(4,0.05,12)}$ = 3.33 R_C = 6.7400

r = 5 : $Q'_{(5,0.05,12)}$ = 3.36 R_C = 6.8010

r = 6 : $Q'_{(6,0.05,12)}$ = 3.40 R_C = 6.8820

$Y_{(1)}$	= Unfermented soybean	: 59.65 ^{c}
$Y_{(2)}$	= Soybean residue tooa-nao	: 64.65 ^{bc}
$Y_{(3)}$	= Soybean tooa-nao	: 66.92 ^{b}
$Y_{(4)}$	= Unfermented soybean residue	: 67.68 ^{b}
$Y_{(5)}$	= Soybean tempeh	: 68.18 ^{b}
$Y_{(6)}$	= Soybean residue tempeh	: 74.95 ^{a}

VITA

Miss Orawan Keeratisuthisathorn was born on May 18, 1967 in Supanburi, Thailand. She received her Bachelor of Science in Pharmacy in 1991 from the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. She admitted with the degree of Master of Science in Pharmacy, Chulalongkorn University in 1994.