

REFERENCES

- Abe, A., Inoue, A., Osami, R., Moriya, K. and Horikoshi, K. (1995). Properties of newly isolated marine bacterium that can degrade polyaromatic hydrocarbons in the presence of organic solvents. Journal of Marine Biotechnology 2: 182 - 186.
- Aono, R., Doukyu, N., Kobayashi, H., Nakajima, H. and Horikoshi, K. (1994). Oxidative bioconversion of cholesterol by *Pseudomonas* sp. strain ST-200 in a water-organic solvent two-phase system. Applied and Environmental Microbiology 60(7): 2518-2523.
- Aono, R. (1998). Improvement of organic solvent tolerance level of *Escherichia coli* by overexpression of stress-responsive genes. Extremophiles 2(3): 239-48.
- Bernal, P., Munoz-Rojas, J., Hurtado, A., Ramos, J. L. and Segura, A. (2007). A *Pseudomonas putida* cardiolipin synthesis mutant exhibits increased sensitivity to drugs related to transport functionality. Environmental Microbiology 9(5): 1135-45.
- Bohin, J. P., Rigomier, D. and Schaeffer, P. (1976). Ethanol sensitivity of sporulation in *Bacillus subtilis*: a new tool for the analysis of the sporulation process. Journal of Bacteriology 127: 934-940.
- Bustard, M. T., Whiting, S., A. D., Phillip C, C. and Wright (2002). Biodegradation of high-concentration isopropanol by a solvent-tolerant thermophile, *Bacillus pallidus*. Extremophiles 6: 319-323.

- Cruden, D. L., Wolfram, J. H., Rogers, R. D. and Gibson, D. T. (1992). Physiological properties of a *Pseudomonas* strain which grows with *p*-Xylene in a two-phase (organic-aqueous) medium. Applied and Environmental Microbiology 58(9): 2723 - 2729.
- de Bont, J. A. M. (1998). Solvent-tolerant bacteria in biocatalysis. Extremophiles 16: 493-499.
- Dashti, N., Al-Awadhi, H., Khanafer, M., Abdelghany, S. and Radwan, S. (2008). Potential of hexadecane-utilizing soil-microorganisms for growth on hexadecanol, hexadecanal and hexadecanoic acid as sole sources of carbon and energy. Chemosphere 70: 475 - 479.
- Dulley, J. R. and Grieve, P. A. (1975). A simple technique for eliminating interference by detergents in the lowry method of protein determination. Analytical Biochemistry 64: 136-141.
- Faizal, I., Dozen, K., Hong, C. S., Kuroda, A., Takiguchi, N., Ohtake, H., Takeda, K., Tsunekawa, H. and Kato, J. (2005). Isolation and characterization of solvent-tolerant *Pseudomonas putida* strain T-57 and its application to biotransformation of toluene to cresol in a two-phase (organic-aqueous) system. Journal of Industry Microbiology Biotechnology 32: 542 - 547.
- Fang, Y., Lu, Z., Lv, F., Bie, X., Lui, S., Zhongyang, D. and Weifeng, X. (2006). A newly isolated organic solvent tolerant *staphylococcus saprophyticus* M36 produced organic solvent-stable lipase. Current Microbiology 53: 510-515.
- Hayashi, S., Kobayashi, T. and Honda, H. (2003). Simple and rapid cell growth assay using tetrazolium violet coloring method for screening of organic solvent tolerant bacteria. Bioscience and Bioengineering 96(4): 360 - 363.

- Heipieper, H. J., Diefenbach, R. and Keweloh, H. (1992). Conversion of *cis* unsaturated fatty acids to *trans*, a possible mechanism for the protection of phenol-degrading *Pseudomonas putida* P8 from substrate toxicity. Applied and Environmental Microbiology 58(6): 1847-52.
- Heipieper, H. J., Meinhardt, F. and Segura, A. (2003). The *cis-trans* isomerase of unsaturated fatty acids in *Pseudomonas* and *Vibrio*: biochemistry, molecular biology and physiological function of a unique stress adaptive mechanism. FEMS Microbiology Letters 229(1): 1-7.
- Hermann, M., Fayolle, F., Marchal, R., Podvin, L., Sebald, M. and Vandecasteele, J. P. (1985). Isolation and characterization of butanol-resistant mutant of *Clostridium acetobutylicum*. Applied and Environmental Microbiology 50: 1238-1243.
- Hirayama, H., Takami, H., Inoue, A. and Horikoshi, K. (1998). Isolation and characterization of toluene-sensitive mutants from *Pseudomonas putida* IH-2000. FEMS Microbiology Letters 169(2): 219-25.
- Ingram, L. O. (1976). Adaptation of membrane lipids to alcohols. Journal of Bacteriology 125(2): 670-8.
- Ingram, L. O. and Buttke, T. M. (1984). Effects of alcohols on micro-organisms. Advances in Microbial Physiology 25: 253-300.
- Inoue, A. and Horikoshi, K. (1989). A *Pseudomonas* thrives in high concentrations of toluene. Nature 338: 264 - 266.
- Inoue, A. and Horikoshi, K. (1991). Estimation of solvent-tolerance of bacteria by the solvent parameter Log *P*. Journal of Fermentation and Bioengineering 71: 194-196.

- Inoue, A., Yamamoto, M. and Horikoshi, K. (1991). *Pseudomonas putida* which can grow in the presence of toluene. Applied and Environmental Microbiology 57(5): 1560 - 1562.
- Isken, S. and de Bont, J. A. (1996). Active efflux of toluene in a solvent-resistant bacterium. Journal of bacteriology 178(20): 6056-8.
- Isken, S. and de Bont, J. A. (1998). Bacteria tolerant to organic solvents. Extremophiles 2(3): 229-38.
- Kabelitz, N., Santos, P. M. and Heipieper, H. J. (2003). Effect of aliphatic alcohols on growth and degree of saturation of membrane lipids in *Acinetobacter calcoaceticus*. FEMS Microbiology Letters 2: 223-227.
- Kato, C., Inoue, A. and Horikoshi, K. (1996). Isolating and characterizing deep-sea marine microrganisms. TIBTECH 14: 6-12.
- Kim, K., Lee, S., Lee, K. and Lim, D. (1998). Isolation and characterization of toluene-sensitive mutants from the toluene-resistant bacterium *Pseudomonas putida* GM73. Journal of Bacteriology 180(14): 3692-6.
- Lovitt, R. W., Longin, R. and Aeikus, J. G. (1984). Ethanol production by thermophilic bacteria: physiological comparisons of solvent effects on parent and alcohol-tolerant strains of *Clostridium thermohtdrosulfuricum*. Applied and environmental microbiology 48: 171-177.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. Journal of Biological Chemistry 193: 265-275.
- Makin, S. and Beverdge, T. (1996). The influence of a-band and b-band lipopolysaccharide on the surface charateristics and adhesion of *Pseudomonas aeruginosa* to surfaces. Bacteriology 142: 299-307.

- Marchesi, J. R., Sato, T., Weightman, A. J., Martin, T. A., Fry, J. F., Hiom, S. J. and Wade, W. G. (1998). Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Applied and Environmental Microbiology 64: 795-799.
- Matsumoto, M., De Bont, J. A. M. and Isken, S. (2002). Isolation and characterization of the solvent-tolerant *Bacillus cereus* strain R1. Journal of Bioscience and Bioengineering 94(1): 45-51.
- Moriya, K. and Horikoshi, K. (1993). A benzene-tolerant bacterium utilizing sulfur compounds isolated from deep sea. Journal of Fermentation and Bioengineering 76(5): 397-399.
- Moriya, K. and Horikoshi, K. (1993). Isolation of a benzene-toluene bacterium and its hydrocarbon degradation. Journal of Fermentation and Bioengineering 76: 168-173.
- Na, K. S., Kuroda, A., Takiguchi, N., Ikeda, T., Ohtake, H. and Kato, J. (2005). Isolation and characterization of benzene-tolerant *Rhodococcus opacus* strains. Bioscience and Bioengineering 99(4): 378 - 382.
- Neumann, G., Veeranagouda, Y., Karegoudar, T. B., Sahin, O., Mausezahl, I., Kabelitz, N., Kappelmeyer, U. and Heipieper, H. J. (2005). Cells of *Pseudomonas putida* and *Enterobacter* sp. adapt to toxic organic compounds by increasing their size. Extremophiles 9: 163 - 168.
- Neumann, G., Cornelissen, S., van Breukelen, F., Hunger, S., Lippold, H., Löffhagen, N., Wick, L. Y. and Heipieper, H. J. (2006). Energetics and surface properties of *Pseudomonas putida* DOT-T1E in a two-phase fermentation system with 1-decanol as second phase. Applied and environmental microbiology 72(6): 4232-8.

- Nicholson, W. L., Munakata, N., Horneck, G., Melosh, H. J. and Perter, S. (2000). Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbial Molecular Biology Reviews 64: 548-572.
- Nielsen, L. E., Kadavy, D. R., Rajagopal, S., Drijber, R. and Nickerson, K. W. (2005). Survey of extreme solvent tolerance in gram-positive cocci: membrane fatty acid changes in *Staphylococcus haemolyticus* grown in toluene. Applied and Environmental Microbiology 71(9): 5171 - 5176.
- Paje, M. L. F., Neilan, B. A. and Couperwhite, L. (1997). A *Rhodococcus* species that thrives on medium saturated with liquid benzene. Microbiology 143: 2975-2981.
- Ramos, J. L., Duque, E., Huertas, M. J. and Haidour, A. (1995). Isolation and expansion of the catabolic potential of a *Pseudomonas putida* strain able to grow in the presence of high concentrations of aromatic hydrocarbons. Bacteriology 177(14): 3911 - 3916.
- Ramos, J. L., Duque, E., Rodrigueq-Herva, J. J., Godoy, P., Haidour, A., Reyes, F. and Fernandez-Barrero, A. (1997). Mechanisms for solvent tolerance in bacteria. Journal of Biological Chemistry 272: 3887-3890.
- Ramos, J. L., Gallegos, M. T., Marques, S., Ramos-Gonzalez, M. I., Espinosa-Urgel, M. and Segura, A. (2001). Responses of gram-negative bacteria to certain environmental stressors. Current Opinion in Microbiology 4(2): 166-71.
- Ramos, J. L., Duque, E., Gallegos, M. T., Godoy, P., Ramos-Gonzalez, M. I., Rojas, A., Teran, W. and Segura, A. (2002). Mechanisms of solvent tolerance in gram-negative bacteria. Annual Reviews Microbiology 56: 743-68.
- Sardesai, Y. N. and Bhosle, S. (2002). Organic solvent-tolerant bacteria in mangrove ecosystem. Current Science 82(6): 622 - 623.

- Sardesai, Y. N. and Bhosle, S. (2004). Industrial potential of organic solvent tolerant bacteria. Biotechnology Progress 20: 655-660.
- Schwartz, R. D. and McCoy, C. J. (1976). Enzymatic epoxidation: synthesis of 7,8-epoxy-1-octene, 1,2-7,8-diepoxyoctane, and 1,2-Epoxyoctane by *Pseudomonas oleovorans*. Applied and environmental microbiology 31(1): 78-82.
- Schwartz, R. D. and McCoy, C. J. (1977). Epoxidation of 1,7-octadiene by *Pseudomonas oleovorans*: fermentation in the presence of cyclohexane. Applied and environmental microbiology 34(1): 47-9.
- Sikkema, J., de Bont, J. A. M. and Poolman, B. (1995). Mechanisms of membrane toxicity of hydrocarbons. Microbiology reviews 59(2): 201-222.
- Segura, A., Godoy, P., Dillewijn, P. v., Hurtado, A., Arroyo, N., Santacruz, S. and Ramos, J. L. (2005). Proteomic analysis reveals the participation of energy-and stress-related proteins in the response of *Pseudomonas putida* DOT-T1E to toluene. Journal of Bacteriology 187: 5937-5945.
- Sikkema, J., de Bont, J. A. M. and Poolman, B. (1994). Interaction of cyclic hydrocarbons with biological membranes. Biological Chemistry 269(11): 8022 - 8028.
- Sikkema, J., de Bont, J. A. M. and Poolman, B. (1995). Mechanisms of membrane toxicity of hydrocarbons. Microbiology reviews 59(2): 201-222.
- Stoops, J. K., Horgan, D. J., Runnegar, M. T., De Jersey, J., Webb, E. C. and Zerner, B. (1969). Carboxylesterases (EC 3.1.1). kinetic studies on carboxylesterases. Biochemistry 8(5): 2026-33.
- Schwartz, R. D. and McCoy, C. J. (1976). Enzymatic epoxidation: synthesis of 7,8-epoxy-1-octene, 1,2-7,8-diepoxyoctane, and 1,2-Epoxyoctane by *Pseudomonas oleovorans*. Applied and environmental microbiology 31(1): 78-82.

- Schwartz, R. D. and McCoy, C. J. (1977). Epoxidation of 1, 7-octadiene by *Pseudomonas oleovorans*: fermentation in the presence of cyclohexane. Applied and environmental microbiology 34(1): 47-9.
- Stoops, J. K., Horgan, D. J., Runnegar, M. T., De Jersey, J., Webb, E. C. and Zerner, B. (1969). Carboxylesterases (EC 3.1.1). Kinetic studies on carboxylesterases. Biochemistry 8(5): 2026-33.
- Toyama, H., Fujii, A., Matsushita, K., Shinagawa, E., Ameyama, M. and Adachi, O. (1995). Three distinct quinoprotein alcohol dehydrogenases are expressed when *Pseudomonas putida* is grown on different alcohols. J Bacteriol 177(9): 2442-50.
- Tumber, V. P., Robarts, R. D., Arts, M. T., Evans, M. S. and Caldwell, D. E. (1993). The influence of environmental factors on seasonal changes in bacterial cell volume in two prairie saline lakes. Microbial Ecology 26: 9 - 20.
- Unagul, P., Assantachai, C., Phadungruengluij, S., Supphantharika, M., Tanticharoen, M. and Verduyn, C. (2007). Coconut water as a medium additive for the production of docosahexaenoic acid (C22:6 n3) by *Schizochytrium mangrovei* Sk-02. Bioresource Technology 98: 281 - 287.
- Usami, R., Fukushima, T., Mizuki, T., Yoshida, Y., Inoue, A. and Horikoshi, K. (2005). Organic solvent tolerance of halophilic archaea, *Haloarcula* strain: effects of NaCl concentration on the tolerance and polar lipid composition. Journal of Bioscience and Bioengineering 99: 169-174.
- Veeranagouda, Y., Karegoudar, T. B., Neumann, G. and Heipieper, H. J. (2006). *Enterobacter* sp. VKGH12 growing with *n*-butanol as the sole carbon source and cell to which the alcohol is added as pure toxin show considerable differences in their adaptive responses. FEMS Microbiology Letters 254: 48 - 54.

- Weber, F. J., Ooijkaas, L. P., Schemen, R. M. W., Hartmans, S. and de Bont, J. A. M. (1993). Adaptation of *Pseudomonas putida* S12 to high concentrations of styrene and other organic solvents. Applied Microbiology and Biotechnology 59(10): 3502 - 3504.
- Wick, L. Y., Ruiz de Munain, A., Springael, D., Harms, H. and de, M. A. (2002). Responses of *Mycobacterium* sp. LB501T to the low bioavailability of solid anthracene. Applied Microbiology and Biotechnology 58(3): 378-85.
- Williams, T. I., Jennifer, C., Bert, C. C. and Strobel, H. J. (2007). Proteomic profile changes in membranes of ethanol-tolerant *Clostridium thermocellum* Applied and environmental microbiology 74: 422-432.
- Zahir, Z., Seed, K. D. and Dennis, J. J. (2006). Isolation and characterization of novel organic solvent-tolerant bacteria. Extremophiles 10: 129 - 138.

APPENDICES

APPENDIX A

A.1 Gram stain

A.1.1 Dye and chemical solution for bacterial staining

The widely used staining technique in bacteriology is the Gram stain developed in 1884 by Hans Christain Gram. This procedure is a differential stain method meaning that bacteria will give different results depending on their cell wall chemistry. The Gram stain divides bacteria will be purple after the last step; gram negative bacteria will be red. The size, shape and arrangement of the organisms can also be determined from a stained specimen using microscope.

1. Gram’s crystal violet

Solution A	Crystal violet	2	grams
	Ethyl alcohol	20	grams
	Distilled water	100	milliliters
Solution B	Smmonium oxalate	0.8	milliliters
	Distilled water	100	milliliters

Solution A and B were mixed and filtrated in order to remove dregs before using.

2. Gram’s iodine

Iodine	1	grams
KI	2	grams
Distilled water	300	milliliters

KI was dissolved in distilled water and added iodine before using.

3. Gram’s alcohol

Ethyl alcohol	98	milliliters
---------------	----	-------------

oxidase reagent. Observe for a color change. A positive reaction appears pink, then maroon and finally black. Take care to avoid contact with the oxidase reagent.

Catalase test

This test determines bacterial production of catalase enzymes. Place a drop of hydrogen peroxide (3% H_2O_2 -reagent grade) on a microscope slide or in the concave surface of a hanging drop slide. With a sterile loop, collect a sample of 18-24 hour old pure bacterial culture. Place the loop in the hydrogen peroxide. If the test is positive, there will be immediate bubbling or foaming and liberation of O_2 gas. Records results

TSI/ H_2S production

This medium can determine the ability of an organism to utilize specific carbohydrates incorporated in basal growth medium, with or without the production of gas, along with the determination of hydrogen sulfide (H_2S) production. TSI agar contains the three sugars in varying concentration: glucose (1X), lactose (10X) and sucrose (10X). It also contains the pH indicator phenol red. If sugar fermentation occurs, glucose will be initially used and the butt of the tube will be acidic (yellow). After glucose utilization the organism may continue to ferment the remaining sugars. If this occurs the entire tube will become acidic. Certain bacteria are unable to utilize any sugars and will breakdown the peptone present. Peptone utilization causes an alkaline (red) shift in the medium that causes a color changes from orange to red. Blackened medium is caused by hydrogen sulfide production, splitting of the medium or presence of bubble in the butt of the tube can determine gas production. With a sterile needle inoculate the TSI slant by stabbing to the bottom of the tube and then streaking the surface of the slant as

the needle is drawn out of the tube. Screw the cap on loosely. Incubate at 20-24°C and read after 18-24 hours.

RESULTS: A = Acid; K = Alkaline; H₂S = Hydrogen sulfide produced; N = No change

Slant/Butt	Color/Reaction	Interpretation
K/N or K/A	red/orange (oxidative) or red/yellow (fermentative)	only peptone utilized or only glucose-fermented
A/A	Yellow/yellow and/or sucrose-fermented	glucose, plus lactose
Gas	Splitting or bubbles	Gas production
H ₂ S	Black butt	Hydrogen sulfide produced

Motility

This test determines if a bacterial isolate is motile by means of flagella. Place a drop of distilled water or sterile PBS onto the center of a clean microscope cover glass. Place an additional tiny drop in one corner of the cover glass (to adhere the cover glass to the depression slide when it is inverted). Incubate the center drop from a pure stain culture that is 24-48 hours old using a sterile loop. Carefully invert the cover glass and place over the concave portion of a hanging drop slide. Observe for motility using phase contrast at 400x magnification on a compound microscope. Care should be taken to not interpret “drift” or “Brownian motion” as motility. Record results as motile or non-motile.

If this method fails to show motility then inoculate a nutrient broth with the isolate and incubate at room temperature until growth is obtained usually 24 hours. After incubation use a sterile loop or sterile dropper and place a drop on a clean cover glass. Place a tiny drop of distilled water in one corner of the same cover glass. Continue as above. Semi-solid motility test medium can be used. Stab the medium with a small amount of inoculum. Incubate overnight at room temperature. If the bacterial species is motile, the medium will become turbid with growth that radiates from the line of inoculum. If the bacterial species is non-motile, only the stab line will have visible bacterial growth.

Citrate Utilization

Organisms that are able to use citrate as the sole source for metabolism and growth are able to grow on Simmons citrate agar. By metabolizing citrate by the bacteria alkaline conditions are formed in the medium. The pH indicator in on Simmons citrate agar, bromothymol blue, will turn from green from acidic conditions to a royal blue when the medium becomes alkaline.

Inoculate the Simmons Citrate agar by making a streak onto the surface of the slant with an 18-24 hour old pure culture. Incubate for up to 4 days at 20-24°C.

RESULTS: Positive-growth and medium color change to a blue-green or royal blue.

Negative-little or no growth and no color change in the medium, remaining dark green.

Urease test

Inoculate the surface of a Christensen urea agar slant and incubate. A positive test is indicated by a red-violet color

Nitrate reduction

To determine the ability of an organism to reduce nitrate (NO_3) to nitrite (NO_2) or further reduced products. Inoculate the Nitrate Broth with 18-24 hour old pure culture. Incubate 24-48 hour at 20-24°C aerobically. After incubation add about 5 drops of α -naphthylamine and sulfanilic acid, add a small amount of zinc dust.

RESULTS: Positive-Formation of a pink or red color in the medium within 1-2 minutes following the addition of naphthylamine and sulfanilic acid or no color development within 5-10 minutes after adding zinc dust.

Negative-No pink or red color development within 1-2 minutes following the addition of naphthylamine and sulfanilic acid or red color development within 5-10 minutes after adding zinc dust.

Esculin hydrolysis

To determine the ability of an organism to hydrolyze the glycoside esculin (aesculetin) to esculetin (aesculetin) and glucose in the presence of bile (10 to 40%). Inoculate the surface of the bile esculin slant with inoculum from an 18 to 24 hour old pure culture. Incubate 20-24°C for 24 to 48 hour.

RESULTS: Positive-Presence of black to dark brown color on the slant.

Negative-No blackening of the medium.

Voges-Proskauer reaction

To detect the production of acetylmethylcarbinol (atoin), a natural product formed from pyruvic acid in the course of glucose fermentation. Inoculate buffered glucose broth with the organism and incubate at 37°C for 3 days. Add approximately 3

ml of alpha naphthol, followed by 1 ml of 40% KOH. Mix well and allow to stand for 30 minutes.

RESULTS:

Pink	VP(+)
No change	VP(-)

Malonate Utilization

A method to establish if a bacterial isolate is able to utilize sodium malonate as its only source of carbon. Inoculate malonate broth with a light inoculum from an 18 to 24 hour pure culture. Incubate 24-48hours at 20-24°C.

RESULTS: Positive-Light blue to deep blue color throughout the media

Negative-Color remains the same as un-inoculated tube-green

Gelatinase Liquefaction

A test to determine bacterial production of gelatinase enzymes that liquefy gelatin. Inoculate by stabbing ½ to 1 inch deep into the nutrient gelatin media with a heavy inoculum from an 18 to 24 hour pure culture. Incubate 18 to 24 hours at 20-24°C.

RESULTS: Positive-Media is liquefied. Weak results can be visualized by rapping the tube against the palm of the hand to dislodge droplets of liquid from the media. Any drops seen are considered positive.

Negative-No liquefaction occurs in media.

Carbohydrate fermentation test

Bacteria metabolize carbohydrates by oxidative and / or fermentative pathways. Oxidation occurs in the presence of atmospheric oxygen (aerobic), whereas fermentation takes place in an anaerobic environment. Metabolism of the carbohydrate dextrose by either an aerobic or anaerobic pathway results in acid production. The resulting acidic environment causes the Brom Thymol blue pH indicator in the medium to turn from green to yellow. The presence of bubbles in the tube indicates gas production(aerogenic). If no reaction occurs, the medium can remain unchanged or become alkaline (blue at the surface). A deep butt tube (~7 ml in 16×125mm) is used for this test. With a sterile needle to a small inoculum from an isolated colony and stab to the bottom of the tube. Incubate at 20-24°C for 24-48 hour. Check tubes at 24 hour for acid and /or gas production.

RESULTS: A = acid (yellow); AG = acid + gas, N = no change or alkaline

	Top of Tube	Bottom of Tube
Oxidative	A	N
Fermentative	AG or A	AG or A
Non-reactive	N	N

Decarboxylase test (Lysine or Ornithine)

A determination of bacterial enzymatic capability to decarboxylate an amino acid to form an amine with resultant alkalinity. For each isolate to be tested, it is necessary to inoculate a decarboxylase control tube and lysine or ornithine test tube. Use light inoculum from 18 to 24 hour pure culture. Add 1 to 2 ml oil overlay to each tube.

Incubate 24 hours at 20-24 °C. A prolonged incubation or up to four days may be necessary.

RESULTS:

<u>Test Result</u>	<u>Lysine or ornithine Tube</u>	<u>Control Tube</u>
Positive	Turbid to faded purple (glucose fermented, Decarboxylase produced)	Yellow (glucose fermented)
Negative	Yellow (glucose fermented, Decarboxylase not produced)	Yellow (glucose fermented)
Negative	Purple (glucose not fermented, Decarboxylase not produced)	Purple (glucose not fermented)

Arginine Dihydrolase

This method is based on the direct measurement of the disappearance of arginine. Make a dense suspension of the bacteria in 0.033 mM phosphate buffer (pH 6.8). Purge 4 ml of the suspension by bubbling nitrogen through the suspension for several minutes, and add 1 ml of 0.001 M L-arginine monohydrochloride. After purging again, stopper the tubes, incubate them for 2 hours, and heat them at 100 °C for 15 min. After removing the cells by centrifugation, determine the concentration of arginine in the supernatant by the method of Rosenberg et al. as follows. Mix 1 ml of sample with 1 ml of 3 N NaOH, 2 ml of developing solution, and 6 ml of water; read the tubes at 30 min against a blank prepared without arginine by using a colorimeter equipped with a green filter (540nm); and compare the reading with those obtained with an uninoculated control containing arginine.

RESULTS: A positive test is indicated by the disappearance of some or all of the arginine.

A.3 Preparation of *E. coli* competent cells for heat shock method

Inoculate 100 ml sterile LB medium with 200 μ l overnight culture of *E. coli* DH5 α then grown at 37°C, 250 rpm until OD600 reach 0.3-0.4. The culture was incubated on ice for 30 minutes. The cells were collected by centrifugation at 5,000 rpm, 4°C for 10 minutes. A supernatant was discarded. The pellet was resuspended with equal volume of cold 0.1 M MgCl₂ and centrifuge at 5,000 rpm, 4°C for 10 minutes. A supernatant was discarded then the pellet was resuspended in 10 ml cold 0.1 M CaCl₂ and incubated on ice for 30-60 minutes. After incubation, the cell suspension was centrifuge at 5,000 rpm, 4°C, for 10 minutes. A supernatant was discarded then the pellet was resuspended in 1.8 ml cold 0.1 M CaCl₂ and 0.4 ml sterile glycerol. The cell suspension was aliquot 100 μ l / tube on ice prior to immediate drop into liquid nitrogen. The competent cells were stored at -80°C until use.

The plasmid was mixed with cold cell suspension in microtube and place on ice for 1 hour. The mixture was incubated at 42°C for 90 seconds and placed on ice for 5 minutes. The cell suspension was transferred into a new sterile tube containing 1 ml of LB broth. The transformed cells were incubated at 37°C, 200 rpm for 1 hour. The transformed cells were then spreaded onto the LB agar plate containing appropriate antibiotic. The plate was incubated at t 37°C for overnight.

Table A.1 The biochemical test result of four bacterial isolates were chosen

Biochemical Tests	Bacterial isolates			
	No.45	No.4/1	No.13	No.27
Gram stain	+ bacilli	+ bacilli	+ bacilli	+ cocci
Hemolysis	β	β	β	
Anaerobic growth		+		
TSI	K/A	K/A	K/K	N/N
H ₂ S				-
Catalase	+	+	+	+
Oxidase	+	+	+	+
Motility	+	+	+	-
Indole				
Citrate	+	+	-	-
Urease	+	+	-	-
Nitrate	+	+	-	-
N ₂ Gas				-
Esculin				-
42°C(Growth)				
Acetate				
VP	+	+	-	+
Gelatinase	+	+	+	+w
Egg Yolk	-	+	-	
Glucose/Gas	+	+	+	
Lactose				-
Maltose				
Mannitol	+	-	+	-
D-Xylose	+	-	-	-
Sucrose				
L-Arabinose	+	-	-	
Fructose				-
Trehalose	+	+	+	
Starch hydrolysis	+	+	+	+
LDA		-		
Lysine				
Arginine	-		-	-
Ornithine		-		
Alkaline phos.				
Camp test				
Flagella				
Bacterial identification	<i>Bacillus subtilis</i>	<i>Bacillus cereus</i>	<i>Bacillus circulans</i>	<i>Dienococcus sp.</i>

APPENDIX B

Esterase activity assay

Principle:



Condition:

Temperature = 25°C, pH = 8.0, $A_{420\text{ nm}}$, Light path = 1 cm

Method: Continuous spectrophotometric rate determination

Reagents:

A. 50 mM potassium phosphate buffer, pH 8.0 at 25°C

(Prepare 100 ml in deionized water using potassium phosphate, monobasic, anhydrous and adjust pH 8.0 with 1 M of KOH.)

B. 100 mM *p*-nitrophenyl acetate (PNPB)

(Prepare 10 ml in dimethylsulfoxide using *p*-nitrophenyl acetate)

C. Esterase enzyme solution

(Immediately before use, prepare a solution containing 0.5-1.0 unit/ml of esterase in cold reagent A.)

Procedure:

Pipette (in milliliter) the following reagents into suitable cuvettes:

	<u>Test</u>	<u>Blank</u>
Reagent A (buffer)	0.99	0.99
Reagent B (PNPB)	0.01	0.01

Mix by inversion and equilibrate to 25°C. Monitor the $A_{420\text{ nm}}$ until constant, using a suitably thermostatted spectrophotometer. Then add:

	<u>Test</u>	<u>Bank</u>
Reagent A (buffer)	0.10	-
Reagent B (PNPB)	-	0.01

Immediately mix by inversion and record the increase in $A_{420\text{ nm}}$ for approximately 5 minutes. Obtain the $\Delta A_{420\text{ nm}}/\text{minute}$ using the maximum linear rate for both the test and blank.

Calculations:

$$\text{Units/ml enzyme} = \frac{(\Delta A_{420\text{ nm}}/\text{min test} - \Delta A_{420\text{ nm}}/\text{min bank}) (1) (\text{df})}{(12.95) (0.1)}$$

1 = Total volume (in milliliters) of assay

df = Dilution factor

12.95 = Millimolar extinction coefficient of *p*-nitrophenol at 420 nm at pH 8.0

0.1 = Volume (in milliliter) of enzyme used

$$\text{Units/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

Unit definition:

One unit will hydrolyze 1.0 μmole of *p*-nitrophenyl acetate and *p*-nitrophenol per minute at pH 8.0 at 25°C.

Final assay concentration:

In a 1 ml reaction mix, the final concentrations are 50 mM potassium phosphate, 1 mM *p*-nitrophenyl acetate and 0.05-0.10 unit of esterase.

APPENDIX C

Bacterial volume and surface of gram-positive bacilli (Neumann *et al.*,2005)

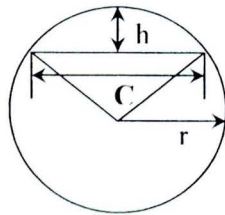
Cells dimensions of cylindrical bodies were directly measured from the TEM photographs to calculate cell volume and cell surface area by the following equations:

$$V (\mu\text{m}^3) = r^2\pi h,$$

$$A (\mu\text{m}^2) = 2r^2\pi + 2\pi rh,$$

where r is the radius and h the length of the cylindrical cells. Average cellular volumes and surface areas were calculated by using 30 individual bacteria per population. Cells showing deformations depressions were not considered.

Bacterial volume and surface of gram-negative cocci (Tumber *et al.*,1993)



$$V = 2 \pi r^3 h = 2.0944 r^2 h$$

$$A = 3.1416 r (2 h + 1c)$$

$$c = 2 \sqrt{h (2 r - h)}$$

BIOGRAPHY

Miss Ajiraporn Kongpol was born on June 1982, in Krabi province, Thailand. She received Bachelor's Degree in Applied biology and Microbiology, Faculty of science, Suan Sunandha Rajaphat University in 2005. She Master degree study in the Biotechnology Program, Faculty of science, Chulalongkorn University, Bangkok, Thailand in June 2005. She finished Master Degree of science in Biotechnology Program in May 2008. During her study, the output of her research work can be summarized as follows:

1. **Ajiraporn Kongpol**, Junichi Kato, and Alisa S. Vangnai. (2008) Isolation and characterization of *Deinococcus geothermalis* T27, a thermophilic and organic solvent-tolerant bacterium able to grow in the presence of high concentrations of ethyl acetate. *Submitted to FEMS Microbiology Letters*
2. Alisa S. Vangnai, **Ajiraporn Kongpol**, Thitikamon Sitthisorn, and Junichi Kato. (2007) Biotransformation of indole using thermo-tolerant, organic solvent-tolerant bacteria. Asia Pacific Biochemical Engineering Conference, Taiwan, November 4-7.
3. **Ajiraporn Kongpol**, Junichi Kato, and Alisa S. Vangnai. (2007) Characterization of thermo-tolerant, organic solvent-tolerant bacteria for industrial biotechnology application JSPS-NRCT Concluding Joint Seminar on Development of Thermotolerant Microbial Resources and their Application. The 33rd Congress on Science & Technology, Thailand (STT33) Science and Technology for Global Sustainability. Walailak University, Nakhon Si Thammarat, Thailand. October, 18-20.



