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THESIS

SCREENING AND ISOLATION OF HEAVY METALS RESISTANT-BIOPOLYMER PRODUCING BACILLUS STRAINS USING AS A HEAVY METAL BIOSORBENT

RUADEERAT BOONSANIT

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Ruadeerat Boonsanit 2012: Screening and Isolation of Heavy Metals Resistant-Biopolymer Producing *Bacillus* Strains Using as A Heavy Metal Biosorbent**.** Master of Science (Bioproduct Science), Major Field: Bioproduct Science, Division of Science. Thesis Advisor: Orawan Chunhachart, Ph.D. 104 pages.

Heavy metals resistant-biopolymer producing bacteria were screened and isolated from fermented soybean, soil and sludge. Examination of these isolates for heavy metals tolerance was carried out in term of growth and survival in medium containing Pb(II) and Cd(II). The isolated bacteria were characterized and identified as genus *Bacillus* based on morphology and biochemical test. *Bacillus* sp. CR002 is able to tolerate high concentration of Pb(II) (700 mg/L) in minimal salt medium. *B. subtilis* MS104 is the most resistance to Cd(II) among all isolates (20 mg/L). Whereas, the amount of biopolymer produced by *B. subtilis* NT147 is the highest among all isolates. Characterized of the biopolymer produced from isolate NT147 revealed that glutamic acid was a main component of the biopolymer consisting of D- and L- glutamic acid ratio of 61:39. Biomass of CR002, MS104 and biopolymer produced from the isolate NT147 were selected for further studies. Biosorption of Pb(II) and Cd(II) by γ-PGA produced from NT 147 and biomass of CR002 and MS104 were investigated and effects of pH, temperature, initial metal concentration and biomass concentration were also studied. The maximum Pb(II) and Cd(II) sorption were found at pH 5. The removal efficiency of Pb(II) by biopolymer from NT147 and biomass of CR002 were 85 and 84 %, respectively. In addition, Cd(II) adsorption onto biomass of MS104 and biopolymer from NT 147 had the maximum adsorption capacity at 29 and 88% Cd(II) removal, respectively. The results indicate that temperature does not affect on adsorption capacity over temperature range of this study. Whereas, the highest percentage removal for lead and cadmium by biomass cell were obtained from 1.0 g dried biomass/L. The adsorption data results similarly fit with empirical models adsorption isotherm of Langmiur.

Student's signature | Thesis Advisor's signature | / /

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Ruadeerat Boonsanit
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ABBREVIATIONS

b_L = Langmiur isotherm constant (L/mg)

C = Concentration of ion metals at time (mg/L)

°C = Degree Celsius

C_0 = Initial concentration of ion metals (mg/L)

Ceq = Concentration of ion metals at equilibrium (mg/L)

K_F = Freundlich constant characteristic of the system related to the adsorption capacity ($\mathrm{mg}^{(n-1)/n}\ \mathrm{L}^{1/n}/\mathrm{g}$)

M = Mass of biosorbent (g)

n = Freundlich constant characteristic of the system related to the adsorption intensity

Q = Metal uptake (mg/g)

q_{eq} = Metal uptake at equilibrium (mg/g)

q_{max} = Saturated adsorption capacity (mg/g)

R^2 = correlation coefficient

$\mathrm{R_L}$ = Separation factor

V = Volume of metal solution (ml)

SCREENING AND ISOLATION OF HEAVY METALS RESISTANT- BIOPOLYMER PRODUCING BACILLUS STRAINS USING AS A HEAVY METAL BIOSORBENT

INTRODUCTION

Heavy metals contamination of soil is widespread (Giller *et al*., 1998). The toxic heavy metals pollutants such as cadmium, lead, arsenic and copper gain entry into agricultural lands through irrigation water contaminated with untreated mining and industrial effluents, sewer water, composes and fertilizers (Jabeen *et al*., 2009). These metals are highly toxic to plants, animals and humans, arriving from the food chain and difficult to remediate. Although they are non-essential element, they are readily absorbed by plant roots, probably complete with other bivalent ions, and are then accumulated inside the organs and grains. The phytotoxicity of cadmium on seed germination, plant growth, plant development, and physiology is well established (Wahid *et al*., 2009). Whereas human excessive long-term exposure to cadmium produces a wide variety of toxic effects and the critical effect is severe damage of kidney (Friberg, 1984). Furthermore, heavy metals contamination can have significant effects on indigenous microbial populations. For example, heavy metals may reduce species composition and limit microbial reproduction. Heavy metals have also been shown to affect microbial activity, such as nitrogen fixation in rhizobia (El-Aziz *et al*., 1991). Each heavy metal has unique biofunctions or biotoxicities. For example, copper can enhance microbial growth at low concentrations but suppress growth at high concentrations (Wu *et al*., 2006). In contrast, cadmium has high toxicity at low concentrations.

In polluted environment, heavy metals resistant bacteria develop their mechanisms to confer resistant to these heavy metals such as precipitation of heavy metals as phosphates, carbonates, and sulfide metals, exopolymers, energy-dependent metal efflux system, and intracellular sequestration with low molecular weight cystein rich proteins (Gadd, 1990; Hughes and Poole, 1989; Silver, 1998). Several bacteria are capable to produce exopolymer such as *Bacillus megaterium* and *B. subtilis* that is able to produce biopolymer composed of D-, L-glutamic acid (Gadd, 1990; Shih and Van, 2001). *Pseudomonas marginalis* also produces exopolymer surrounding cell envelope to decrease lead toxicity (Roane, 1999). While some bacteria possess plasmids and genes encoding for specific proteins and enzymes involved in resistance of toxic heavy metal including Ag, Cd(II), Co(II), Cr(II) Cu(II), Hg(II), Ni(II), Pb(II), Sb(III) and Zn(II) (Tsai, 1992).

Conventional methods of metals treatment such as precipitation, coagulation, membrane process and carbon adsorption, as well as ion exchange are ineffective at metal concentrations less than 100 mg/L (Zhang *et al*., 2005; Yavuz *et al*., 2006). For over a decade, phytoremediation and biosorption using biomass of microorganisms were evaluated as an alternative to exclude heavy metals from environment (Kapoor and Viraraghavan, 1995; Sarand, 2002). Microbial surfaces have several functional groups that can react with metal ions (Kang *et al*., 2007). In recent year, biopolymers derived from microorganisms have been emerging as an alternative treatment for scavenging heavy metals from aqueous systems (Wingender *et al*., 1999; Liu and Fang, 2002). γ-Polyglutamic acid (γ-PGA) is an edible, high molecular mass (10 to 2,000 kDa) bipolymer that consists of D- and L-glutamic acid polymerized through γ-glutamyl bonds. γ-PGA is produced by certain *Bacillus* strains as a capsular or an extracellular viscous material (Shih and Van, 2001). Because of it’s biocompatible to nature; γ-PGA and its derivatives have been of the interest in various fields such as food, cosmetics, agriculture, water treatment and bioremediation (Shih and Van, 2001). γ-PGA has been reported to be an effective bio-sorbent for cationic dyes (Inbaraj *et al*., 2006) and several metal ions; Ni (II), Cu(II), Mn(II), Al(III) and Cr (III) (McLean *et al*., 1990 ; 1992), Hg(II) (Inbaraj *et al*., 2009), Pb(II) and Cd (II) (Mark *et al*., 2006) in water treatment. However, detoxification of heavy metals tainted in soil by γ-PGA or γ-PGA producing strains in order to protect plant from biotoxicity is still unrevealed.

γ-PGA producing strains that tolerate to high concentration of heavy metals may survive and prolong their biosorption activity in heavy metals contaminated soil. Thus, it is possible that γ-PGA or γ-PGA producing strains may play a significant role in the restoration of heavy metals contaminated soil. Therefore, the objectives of this work were to isolate heavy metals resistant *Bacillus* strains that are capable to produce γ-PGA from Thai Thua-nao, heavy metals contaminated soil and sludge to use as heavy metal bio-sorbent. The heavy metals adsorption efficiency of purified γ-PGA and γ-PGA producing cells were also be studied.

OBJECTIVES

1. To screen and isolate γ-polyglutamic acid (γ-PGA) producing *Bacillus* strains that tolerate toward hazardous heavy metals such as cadmium (Cd) and lead (Pb) from Thai Thua-nao, heavy metals contaminated soils and sludge.

2. To compare the heavy metals adsorbtion efficiency between purified γ-PGA of *B. subtilis* NBRC16449, biopolymer produced isolated and cells of the selected strains.

LITERATURE REVIEW

1. Heavy metals and theirs occurrences

Heavy metals contamination in soil is a major problem for the environmental quality of the world (Purves, 1999; Yoon *et al*., 2006; Makino *et al*., 2006). It has become increasingly serious with the development of mine exploration, metallurgy industry, paint pigments and irrigation of wastewater. For over a decade, some countries have faced cadmium contamination problems including accumulation of cadmium in staples and sea animals and health problems of people due to cadmium long-term exposure and they have tried to mitigate these circumstances.

1.1 Cadmium

Cadmium is a silver-white, ductile, very malleable metal and its surface has a bluish tinge and the metal is soft enough to be cut with a knife. It is soluble in acids but not in alkalis. It is similar in many respects to zinc but it forms more complex compounds. Cadmium is a one major of metal significantly in the industries. Its compounds are used in silver-zinc storage batteries, in Ni-Cd batteries, in pigments, in coating and painting, and in plastics to improve high -temperature properties.

1.1.1 Cadmium contamination in the environment

Environmental contamination by cadmium results that about 25,000 tons per year of cadmium was released into soil or river though weathering of rocks, human activities such as manufacturing, mining and agricultural. Sun (2004) reported that nearly 2.0×107 hm2 of cultivated land of China is contaminated with heavy metals and includes 2.8 ×105 hm2 farmland contaminated by cadmium (Cd) (Cao *et al*., 1999). Natural sources of cadmium, including industrial emissions and the application of fertilizer and sewage sludge to farm land and contaminated in soils. Moreover, increased cadmium uptake by crops and vegetables, are well known to enter to food chains and can also have effects for human consumption.

1.1.2 Toxicity of cadmium

Cadmium exposure may cause of cancer, bone fracture, reproductive failure and damage to the central nervous system in human body. The main organs for cadmium accumulated in humans body is kidney, liver and pancreas (Järap, 2003). Symptoms of cadmium to kidney contain tubular dysfunction, evidenced by an increased excretion of low molecular weight proteins or enzymes (Järap *et al.*, 2000). The studied in Europe have shown signs of cadmium induced kidney damage in the general population at urinary cadmium levels around 2–3 μg Cd/g creatinine (Buchet et al., 1990; Järap *et al.*, 2000).

In 1950's, cadmium contamination in Toyama prefecture, Japan, Mitsui Mining and Smelting Co., Ltd. discharged contaminated water with cadmium to Jinzu River. The river was used mainly for irrigation of rice fields, drinking water, washing, fishing and other use. Affect of cadmium poisoning call 'Itai-Itai" disease, causes weak and brittle of bones, coughing, anemia, kidney failure and lead to death.

Similarly, in northwestern Thailand, zinc mining was established in 1977 in Mea Sot district located in Tak province resulting in rapid leaking of cadmium into Mea Tow River and Mea Tow creek which are the main sources of water supply used for ethnic people, agriculture and fishery. There are several reports exhibited that people lived in contaminated area have high urinary cadmium levels (Swaddiwudhipong *et al.*, 2007; Teekasem *et al.*, 2007). Furthermore, cadmium contaminated in for cultivated land had effected to plants growth and yield.

In addition, studies on animal experiments have proposed that cadmium may be a risk factor for cardiovascular disease, but studies of humans have not been able to confirm this (Järap *et al.*, 1998). However, in Japan study of an excess risk of cardiovascular mortality in cadmium-exposed persons with signs of tubular kidney damage compared to individuals without kidney damage was reported (Nishijo *et al.*, 1995).

1.2 Lead

Lead is a one of the three most toxic metals, may be found in the environment (rock, soil, water, air). It has the most damaging effects on human health. It is a toxic heavy metal pollutant of environment from various sources such as burning of coal, effluents from storage battery industries, automobile exhausts, metal plating and finishing operations, fertilizers, pesticides and from additives in pigments and gasoline.

1.2.1 Lead contamination in the environment

In Thailand, reported that upper Maeklong River located in Srisawat district in Kanchanaburi province, lead contamination was first found in 1989 by Office of National Environment Board in which water in retailing ponds adjacent to the flotation plant especially Khliti creek in Ban Khlitilang contained lead at 0.574 mg/L in which is higher than the National Surface Water Quality Standard. Moreover, Cui *et al* (2004) reported that heavily accumulation of Pb(II) in soil and vegetables in Nanning, China resulting in accumulation of Pb(II) in vegetables in range of 0.38-0.45 mg/kg.

1.2.2 Toxicity of lead

Generally, lead was uptake into human body by ingestion and inhalation, and more accumulation in children than adult was observed. Several reports exhibited that accumulation of Pb(II) in agricultural products effects on production yield. In human, Pb(II) toxicity causes reduction in the haemoglobin synthesis, disturbance in the functioning of kidney, joints, reproductive and cardiovascular systems and chronic damage to the central and peripheral nervous systems (Ogwuegbu and Muhanga, 2005).

Acute toxicity of Pb(II) following ingestion of a large amount of Pb(II), there will be entry tissue interaction. The most sensitive system is the hematopoietic system and nervous system. The biosynthesis of hemes in general were deranged by the presence of Pb(II) and inhibited enzyme activities such as delta-aminolevulinic acid dehydratase (ALAD) heme synthase (HS) (Ogunseitan et al., 2000). All active dividing cells are especially sensitive; hence acute intoxication has major potential for GI and renal mucosal damage. In addition there is a high risk of neurological damage.

Conventional methods for removal heavy metals contaminated, chemical precipitation, ion exchange, electro-winning, electro-coagulation, cementation and reverse osmosis. However, as a result of these processes, significant disadvantage, requirement costly equipment, energy, toxic sludge management and require the experts. In addition, new technology for decreased problem of heavy metal contamination and affordable costs to removed pollutant.

2. Bioremediation of heavy metals

Bioremediation is defined as a process for removing hazardous heavy metals from the environment with emphasis on bio-removal aspects (Wu *et al*., 2010). Bioremediation strategies have been purpose as an attractive alternative owing to their low cost and high efficiency. Bioremediation is primarily explored for purifying metal contaminated waste. The ultimate is to use microorganisms and plants in the biosorption of metals-polluted water, waste stream and soils. However, the described examples are still mainly tested at a laboratory scale and it remains to be seen whether bioremediation will be an industrially acceptable concept (Mejáre and Bülow, 2001).

2.1 Phytoremediation

Phytoremediation describes the treatment of environmental problems through the use of plants and seaweeds to mitigate heavy metals contamination without the need to excavate the contaminant material and dispose of it elsewhere. Possible mechanisms of phytoremediation to remove heavy metal from environment are binding to the cell wall, reduced transport across the cell membrane and chelation (Mejáre and Bülow, 2001). Seaweeds and several plants such as cone, pine, and rice husk have been used as biosorbents to remove heavy metal ions from wetland and soil (Azab *et al*., 1995; Aderhold *et al*., 1996; Volesky *et al*., 1999; Nuhoglu and Oguz., 2003.

Volesky *et al* (1999) extensively studied on seaweed name Sargassum sp. They compared three species of non-living Sargassum biomass for their specific uptake cadmium and copper from aqueous solution. Similarly seaweeds such as Ecklonia maxima, Lessonia flavicans and Durvillea potatorum were reported to be sequester of copper, nickel, zinc, lead, and cadmium ions from solution (Aderhold *et al*., 1996). Besides, dried bagase, rice husk and fermented baggase were also capable to remove cyanide from industrial effluent (Azab *et al*., 1995). Waste biomass such as wheat stem and babul bark was used to remove nickel from effluent of an electroplating industry (Verma and Shukla, 2000). Furthermore, the cone biomass *Thuja orientalis* exhibited high adsorption capacity for removal of copper from waste water (Nuhoglu and Oguz, 2003). Biosorption of chromium using biomass of *Pinus sylvestris* was rapidly with 84% of the total adsorption occurring in 2 hr. at C0 = 150 mg/L and m = 1 g/L which the highest capacity achieved at pH 1.0 (Ucun *et al*., 2002).

Thus, phytoremediation technology are recently becoming recognized as an environmental friendly, cost-effective methods for remediating sites contaminated with toxic metals and possible commercialization of this technology will use in environment treat. However this technology still has some disadvantages blocking widespread i.e. relatively long time for metal accumulation, leaching of metals out of the root zone and critical nutritional conditions requirement and proper soil characteristics (Felix, 1997).

2.2 Microbial remediation

Microbial remediation uses living-microorganisms and dead biomass to mediate heavy metals bioaccumulation (Volesky, 1995). Other possible heavy metal resistance mechanisms in bacterial are the active efflux pumping of the toxic metals out of the cell or the enzymatic detoxification converting a toxic ion into a less toxic or less available metal ions (Mejáre and Bülow, 2001).

Microbial biosorption was a property of certain types of bioremediation, non-living microbial biomass to bind and concentrate heavy metals from various aqueous solutions. The cell wall structure of algae, fungi and bacteria were found to responsible to adsorbtion of heavy metals (Volesky, 1990; Hu *et al*., 1996; Mameri *et al*., 1999). Copper biosorption by wood rotting fungus *Ganoderma lucidum* showed that protein interaction with metals did not play a significant role in copper(II) uptake (Muraleedharan and Venkobachar., 1990). Biosorption of lead by *Phanerochaete chrysogenum* biomass was strongly affected by pH. The saturated uptake capacity for lead sorption at pH 4 and pH 5 was higher than that of activated charcoal and that reported for some other organisms (Niu *et al*., 1993). Non-living waste biomass of *Aspergillus niger* attached to wheat bran was used as a bio-sorbent for removal of zinc and copper from aqueous solution and metal uptake was found to be a function of the initial metal concentration, biomass loading and pH (Modak *et al*., 1996).

2.3 Adsorption isotherm

The quality of biosorbent is judge according to how much sorbate it can attract and remain in an immobilized form. The solute uptake by the biosorbent can be calculated from differences between the initial quantity of solute added to that contained in the supernatant, which is achieved by using the following equation:

$$Q = \frac{V\,(C_0 - C)}{M} \qquad (1)$$

Where Q is the solute uptake (mg/g); V is the volume of the metal-bearing solution contacted with sorbent (L); C0 and C are the initial and equilibrium (or residual) concentration of metal in solution (mg/L), and M is the mass of biosorbent (g) (Volesky, 2007).

Models have an important role in technology transfer from laboratory- to industrial-scale. Appropriate models can help in understanding process mechanisms, analyze experimental data, prediction of answers to operational conditions and optimization of processes. As an effective quantitative means to compare binding strengths and to design biosorption processes (Volesky and Holan, 1995; Limousin *et al*., 2007)

Several adsorption isotherms originally used for gas phase adsorption are available and rapidly adopted to correlate adsorption equilibrium in heavy metals biosorption. The most widely use among them are Frundlich and Lanmiur equations. The applications of isotherms on biosorbent –supported heavy metals removal were adapted for water and wastewater (Limousin et al., 2007).

5.1 Freundlich isotherm

The Freundlich isotherm was originally empirical in nature, but was later interpreted as sorption to heterogeneous surface or surface supporting sites with various affinities. The Freundlich isotherm can be represented as:

$$Q = K_F C_e^{1/n} \qquad (2)$$

Where, Ce is the equilibrium concentration of heavy metal in the solution (mg/L); KF, corresponds to the binding capacity; n is characterize the affinity between the sorbent and sorbate. Equation (2) can also be expressed in linearized logarithmic form and Freundlich constants can be determined.

5.2 Langmiur isotherm

Another widespread-used model to describe heavy metals sorption to biosorbent is the Langmiur model. The Langmiur equation is limited to monolayer coverage onto a completely homogeneous surface with a finite number of identical sites and with negligible interaction between adsorbed molecules (Aksu and Balibek, 2007) the Langmiur equation can be written in following form.

$$Q = \frac{Q_{max} b_L C_e}{1 + b_L C_e} \qquad (3)$$

Where Q_{max} corresponds to the maximum achievable uptake by a system, b_L is related to the affinity between the sorbete and sorbent and C_e is the equilibrium concentration (mg/L). Thus, for a good biosorbent, a high $Q_{\max}$ and a steep initial isotherm slope i.e., high $Q_{\max}$ and b can be determined from the linear plot of C_{eq}/q_{eq} versus C_{eq}.

To further quantify the adsorption properties of the Langmuir isotherm, a dimensionless separation factor or equibrium parameter (R_L) was used. The separation factor R_L was applied to the data to establish the favourability of the adsorption isotherm (Ho *et al*., 2002).

$$R_L = \frac{1}{1+b_LC_0} \qquad (4)$$

Where, C0 is the initial dye concentration in solution (mg/L) and b is the Langmuir constant (L/mg). The value of RL indicates the type of the isotherm in Table 1

Table 1 The separation factor of the Langmuir isotherm model

R_L	Type of isotherm
$R_L>1$	Unfavorable
$R_L=1$	Linear
$0<R_L<1$	Favorable
$R_L=0$	Irriversible

3. Bacterial heavy metals resistance and their mechanisms

Most of heavy metals released from some toxic chemical elements and their deviated chemical compounds do not functionally involve in any activity for bacterial life. However, bacteria need to develop different mechanisms to confer resistances to these heavy metals. Three generalizations may be made: (a) the specificities of plasmid-determined metal resistances, (b) heavy metal resistance systems have been found on enzymatic detoxification and (c) two general resistant mechanisms were found, efflux pumping and bacteria sequestration (Tsai, 1992).

3.1 Intracellular sequestration and biosorption

Intracellular sequestration is accumulation of metals within the cytoplasm to prevent and exposure to essential cellular component. Metallothionines (MTs) are low molecular weight (6–7 kDa), cysteine-rich proteins and found in animals, higher plants, eukaryotic microorganisms and some prokaryotes. The high metal-binding capacity of MTs has been widely exploited. MTs from various sources have been expressed intracellular in *Pseudomonas* sp., *E. coli, Mycobacterium scrofulaceum* (Mergeay, 1991; Rouch *et al*.,1995; Silver and Phung, 1996; Valls *et al*., 2000). However, not devoid of complications and in many instances there have been problems with the stability and short half-life of the expressed heterologous proteins. The cysteine content of MTs is very high, which might interfere with cellular redox pathways in the cytosol (Mejane and Mulow, 2001).

Heavy metal resistant bacteria have been isolated from several contaminated sources such as soil and sludge (Roane, 1990; Basu *et al*,. 1997; Jiang *et al*., 2008). Hamzah *et al* (2009) screened potential bacteria that tolerate towards copper and cadmium from oil sludge pond. The results showed that the isolates A13 was the most tolerant species toward copper and reduction in the growth was reduced about 55% at 100 mg/kg of copper. Meanwhile, isolate B21 is the most tolerant toward cadmium with 29% reduction in growth at 100 mg/kg of cadmium. The isolated bacteria were tolerant towards heavy metals up to 39 and 134 mg/kg of arsenic and cadmium whereby metals may affect the growth, morphology and activity of bacteria (Lorenz *et al*., 2006). Cadmium is more stable in soil and may exist for a long period thus microorganisms can adapt to its toxicity by activating the tolerance mechanism towards cadmium (Piotrowska-Seget *et al*., 2005).

3.2 Enzymatic detoxification of a metal to a less toxic form

Enzymatically facilitated oxidation, reduction, methylation and alkylation reactions constitute mechanism for microbial metal resistance. Detoxification is usually by enzymatic reduction of the cation to the metal, whereas some heavy metal resistance genes are carried on plasmids, whilst others are chromosomal (Cloate, 2003). Kazy *et al* (1999) found that bacteria have mechanisms to convert copper into salt form and it is being absorbed by intracellular precipitation of the cell. Thus, in naturally polluted environment, bacteria can develop heavy-metal resistance mostly for their survivals, especially a significant their mechanism and enzymatic system to detoxicity. According, the detoxification of Hg(II) to Hg via mercuric reductase from a recombinant *E. coli* (Chang *et al*., 1999). Furthermore, *B. circulans* can accumulate heavy metals from aqueous solution and removed 80% of copper and 44% of cadmium from solution containing 495 mg/L of copper and 492 mg/L of cadmium, respectively (Sahoo *et al*., 1992). Whereas, chromium resistant bacteria isolated from soil samples of iron mineral area are capable of reducing toxic Cr(VI) to less toxic Cr(III) (Cheng and Li, 2009).

3.3 Specificity of plasmid-determined metal resistances and Active transport

Active transport of metals out of cell is one mechanism of bacteria resistance to metal toxicity. Efflux pumps possibly obtain from energy of membrane potential and ATP. Cadmium resistance bacteria have been reported to possess a plasmid gene, *cad A* and *cad C* were encodes a cadmium-specific ATPase that transports cadmium out of bacterial cell. The plasmid-encoded *cad* gene system have been found in several genera, including, *P. putida, B. subtilis* and *Desulfovibrio* sp. (Laddaga *et al*., 1985; Lee *et al*., 2001; Naz *et al*. 2005).

Jiang *et al* (2009) have been made to develop heavy metal resistant bacteria by recombination biotechnology, which increase the efficiency of spontaneous mutation. UV-mutated *B. subtilis* 38 was able to tolerate up to 3 mM of cadmium; whereas the wild type of *B. subtilis* can tolerate up to 0.25 mM. The ability of *Rhodobacter sphaeroides* strain R 26.1 to grow in heavy metals contaminated environment and bacterial cultures were carried out in artificially polluted media, enriched with the metals ion i.e. mercury, copper, zinc, nickel, cobalt, molybdenum and chromium. The bacterium was proven to be highly tolerant to heavy metal exposure, especially towards copper, zinc and molybdenum. In addition nickel and cobalt were found to decrease the cellular content of light harvesting complexes (Giotta *et al*., 2006).

3.4 Extracellular sequestration

Metal resistance based on extracellular sequestration has found in several microorganisms. In eukaryote cell has been reported that *Saccharomyces cerevisiae* reduced absorption of Ni(II) by excrete glutathione (Murata *et al*., 1985). Furthermore, Gadd (2004) demonstrated that siderophores, highly specific Fe(III) ligands and low molecular weight are secreted by several genera to aid Fe assimilation. Not only Fe(III) but also siderophores can complex other metals such as manganese, magnesium and chromium(III) (Birch and Bachofen, 1990).

Moreover, Extracellular polymeric substance (EPS) play a very important role in biosorption of heavy metals which produced by microoranism. EPS include a mixture of polysaccharides, mucopolysaccharides and proteins. They contain functional groups such as carboxyl, phosphoric, amine, and hydroxyl groups, which enable EPS to sequester heavy metals (Zhang *et al*., 2006). Several researchers have been reported that EPS can also adsorb or entrap particulate matter such as precipitated metal sulfides and oxides (Flemming, 1995; Vieira and Melo, 1995).

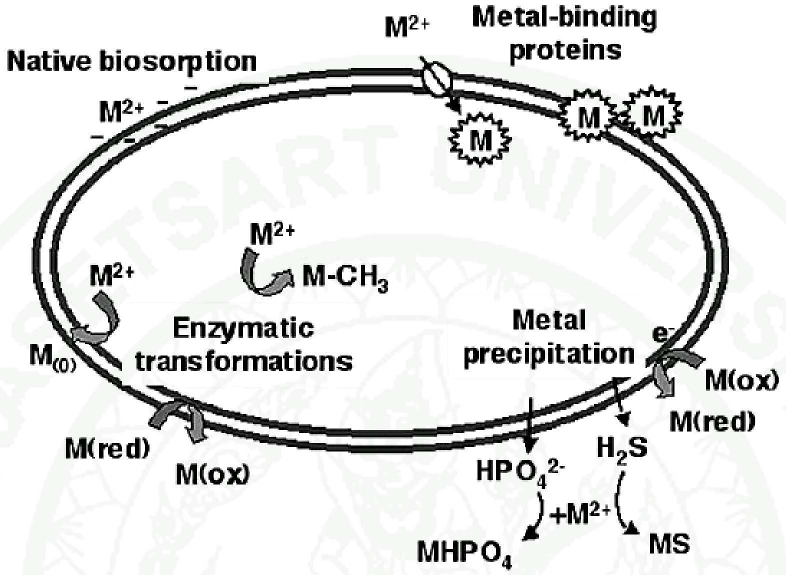


Figure 1 Mechanism of metal absorption by bacterial cell (Valls and Lorenzo, 2002)

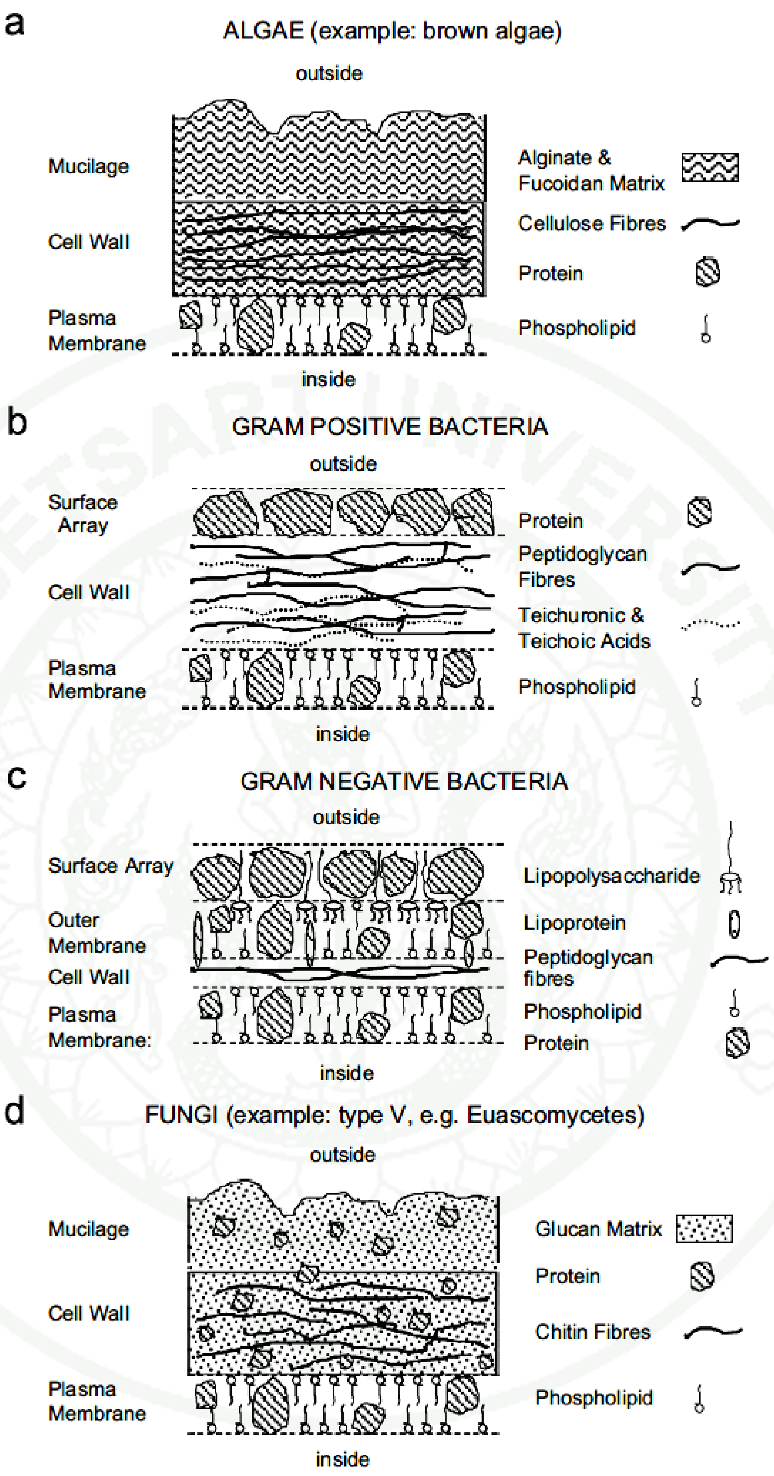
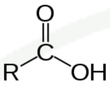
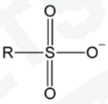
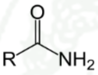
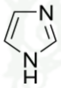
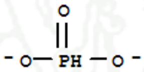
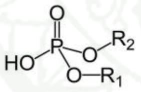


Figure 2 Schematic outline of the cell wall structures of (a) seaweeds; (b) gram+ bacteria; (c) gram⁻ bacteria; (d) fungi (Volesky, 2007)

Table 2 Major of binding groups for biosorption (Volesky, 2007)

Binding group	Structure formular	pKa	HSAB classif.	Ligand atom	Occurrence in selected biomolecule
Hydroxyl	-OH	9.5-1.3	Hard	O	PS, UA,SPS, AA
Carbonyl (ketone)	>C =O	-	Hard	O	Peptide bond
Carboxyl		1.7-4.7	Hard	O	UA, AA
Sulfhyldryl (thio)	-SH	8.3-10.8	Soft	S	AA
Sulfonate		1.3	Hard	O	SPS
Thioether	>S	-	Soft	S	AA
Amine	$-NH_2$	8-11	Int.	N	Cto, AA
Secondary amine	>NH	13	Int.	N	Cti, PG, Peptide bond
Amide		-	Int.	N	AA
Imine	=NH	11.6-12.6	Int.	N	AA
Imidazole		6.0	Soft	N	AA
Phosphonate		0.9-2.1 6.1-6.8	Hard	O	PL
Phosphodiester		1.5	Hard	O	TA,LPS

PS=polysaccharides; UA=uronic acid; SPS= sulfate; PS; CTO= chitosan; PG=peptidoglycan; AA=amino acids; TA=teichoic acid; PL=phospholipids; LPS= lipopolysaccharide

4. γ-Polyglutamic acid and its occurrence

γ- Polyglutamic acid (γ-PGA) is an extracellular biopolymer and unusual anionic polypeptide that made of D-and L- glutamic acid units connected by amide linkages between α- amino and γ-carboxylic acid function groups (Shih and Van, 2001). γ-PGA is secreted through the cell wall, forms capsule during growth, and is also excreted into the culture medium. Because γ-PGA is an extracellular polymer with high molecular mass (MW =100-1,000 kDa) the culture medium becomes highly viscous upon its production. The cell capsulated with γ-PGA possesses negative charges near neutral pH because of the ionization of carboxyl groups in γ-PGA molecules (Do *et al*., 2001).

γ-PGA was first discovered by Ivanovics and co-workers as a capsule of *B. anthracis* which was released into medium upon autoclaving or upon aging and autolysis cells. It is also well known that the mucilage of “natto” (ferment soybeans, a traditional food in Japan) is a mixture of polyglutamic acid and fructan produced by *Bacillus natto* Sawamura (Sawamura, 1913). Furthermore, natto-like fermented soybean foods have also been manufactured rurally in mountainous area of South and Southeast Asia including Nepal, Northern Bengal and Sikkim provinces in India, Bhutan, northern Myanmar, Yunnan province in China and northern Thailand (Thamang *et al*., 1999; Yasuhiro *et al*., 2002; Chunhachart *et al*., 2006).

Thua-nao, a natto-like fermented soybean product which streamed soybeans are wrapped in teak leaves and fermented by natural microorganisms at ambient temperature is produced and consumed widely in northern area of Thailand (Sundhagul *et al*., 1972). In Asia, Thua-nao and natto are very similar in term of proteolytic *Bacillus* fermentation but there was still made by a traditional method with a mixed natural microflora; however, the predominant was *B. subtilis* (Leejeerajumnean, 2003). In phylogenetic trees, the study elucidated the phylogeny of these PGA-producing strains and can be divided into two species, *B. subtilis* and *B. amyloliquefaciens* on natto-like fermented soybean products are manufactured and consumed in many Asian countries (Meerak *et al*., 2007).

Several reports showed that γ-PGA producing *Bacillus* were screened from soil (Ito *et al*., 1996; Xu *et al*., 2005; Chen *et al*., 2005). Furthermore, the other living organisms can produce γ-PGA such as two halophilic eubacteria, *Sporosarcina halophila* and *Planococcus halophila* (Kandler *et al*., 1983) and a halophilic archaebacterium, *Natrialba aegyptiaca* (Hezayen *et al*., 2000). In addition to prokaryotes, the ability of excreting of γ-PGA was also found in eukaryotes such as the nematocysts of *Cynidaria* (Weber, 1990).

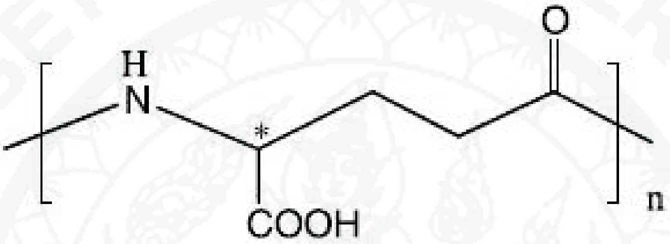


Figure 3 Chemical structure of γ-PGA (Shih and Van, 2001)

4. Heavy metals adsorption by γ-PGA

γ-PGA is water soluble, biodegradable, edible and non-toxic toward humans and the environment. Therefore, potential applications of γ-PGA and its derivatives have been of interest for the past few years in the broad range of industrial fields such as food, cosmetics, medicine and water treatment. γ-PGA and its derivatives (Shih and Van, 2001). Since, the application of γ-PGA is versatile, safe and environmental friendly.

Several studies have been used the γ-PGA adsorption, Bhattacharyya and co-workers discovered a highly innovative and new approach to obtain high metal absorbing microfiltration membrane containing covalently attached PGA with extremely high capacities for heavy metals. Some PGA-functionalized cellulose membranes showed binding capacities as high as 1.3-1.5 g/g membrane with Pb(II) and about 0.8 mol metal sorbed/mol unit for Cd(II) and Ni(II) (Bhattacharyya *et al*., 1998). γ-PGA have been a potential adsorbent for removing Hg(II) from water and wastewater with the maximum binding capacity according to Langmuir isotherm of 96.79 mg/g at pH 6 and 303 K. However, the presence of interfering metals ions could affect the Hg(II) binding on γ-PGA. A multi-site interaction by complex mechanism involving amide and α-carboxylate anion functional groups is proposed for adsorption of Hg(II) by γ-PGA (Inbaraj *et al*., 2009).

Furthermore, biosorption of Cu(II) by γ-PGA obtained from *B. licheniformis* ATCC 9945 under varying conditions of pH, temperature, ionic strength, and in the presence of other heavy metal ions were determined. The Langmuir adsorption isotherm model showed that γ-PGA was able to adsorb Cu (II) at 77.9 mg/g and a binding constant of 32 mg/L (0.5 mM) at pH 4.0, 25°C. Cu(II)-γ -PGA adsorption was relatively temperature independent between 7 and 40°C, while an increase in ionic strength led to a decrease in metal ion binding (Mark *et al*., 2006). The experimental effect of pH, contact time, metals concentration, γ-PGA does and residue essential metals carried out in batch experiment. For experiment showed that optimal pH adsorbtion in pH 2.5 to 5 lead and cadmium can binding on γ-PGA (Siao *et al*., 2009)

Although γ-PGA was reported to be an effective biosorbent for several metal ions, all experiments and industrial application have been done only with water and wastewater. Detoxification of heavy metals tainted in soil by γ-PGA or γ-PGA producing strains in order to protect plant from heavy metal toxicity is still unrevealed. Thus γ-PGA producing strains may a potential tool for the bioremediation of heavy metals contaminated soil.

MATERIALS AND METHODS

Materials

1. SDS-PAGE Apparatus
2. Atomic Absorption Spectrophotometer (AAS) (Varian SpectrAA FS-220)
3. Rotary Shaker
4. Incubator
5. Lyophilizer
6. Hot Plate Stirrer
7. Spectrophotometer
8. Heating Box
9. High performance liquid chromatography (HPLC)
10. γ-PGA produced by *B. subtilis* NBRC 16449
11. Thin layer chromatography paper (Merck, Germany)

Chemicals

1. $Pb(NO_3)_2$ anhydrous (Merck , Germany)
2. $CdCl_2 \cdot 2.5\ H_2O$ (Wako, Japan)
3. Cd standard solution (Nacalai tesque, Japan)
4. Pb standard solution (Merck, Germany)
3. Hydrochloric acid (Q-Rëc, Malaysia)
4. Sodium hydroxide (Ajax Finechem, New Zealand)
5. Acetonitrile (RCI Labscan, Thailand)
6. Butanol (J.T Baker, USA)
7. Acetic acid (RCI Labscan, Thailand)
8. Acetone (J.T Baker, USA)
9. Absolute alcohol (Hayman, England)

Mediums

1. Nutrient agar
2. Luria-Bertani medium
3. PGA production medium
4. Minimal Salt Medium

Methods

1. Bacterial strain

B. subtilis NBRC16449 and all isolated strains were maintain in Luria-Bertani agar slant at 4°C and subcultured monthly. Stock cultures were keep at -70°C.

2. Screening and isolation of γ-PGA producing bacteria

2.1 Screening of γ-PGA producing bacteria from Thua-nao

γ-PGA producing bacteria were isolated from Thua-nao purchased from local markets in northern Thailand. Ten grams of Thua-nao was homogenized in 90 ml of 0.85% NaCl, and the suspension was heated at 80°C for 10 min to destroy vegetative cells. After cooling, the suspension was diluted with 0.85% NaCl and spread onto a nutrient agar plate then the plate was incubated at 37°C for 24 h. Mucoid colonies randomly selected and re-streaked onto a fresh nutrient agar plate until purified colonies were obtained.

2.2 Screening of γ-PGA producing bacteria from soils

Soil sample were randomly collected from 15 to 20 points of a farm land in Mae Sot district. A shovel or spade shovel was used to dig holes as a deep V shape or approximately 15 cm in the vertical. Then, the samples were mixed in the bucket by hands and filled in sample bags. After that soil sample were sun-dried, grind and sieved. Ten grams of soil was homogenized in 90 ml of 0.85% NaCl, and the suspension was heat at 80°C for 10 min to destroy vegetative cells. The suspension was diluted with 0.85% NaCl and spread onto a nutrient agar plate, which incubated at 37°C for 24 h. Mucoid colonies were randomly selected and re-streaked onto a fresh nutrient agar plate until purified colonies were obtained.

All isolates obtained from 2.1 and 2.2 were examined for their ability to produce γ-PGA. Pure culture of the isolated strains were inoculated into LB broth and PGA-production medium then incubated at 30°C with shaking at 120 rpm for 20 h. The culture broth was collected and centrifuged to remove cells. The supernatant was collected and examined for γ-PGA by SDS-PAGE and then the gel was stained by Alcian blue. γ-PGA produced from *B. subtilis* NBRC16449 was used as a standard.

3. Screening of heavy metals resistant strains

Bacterial isolates from soils samples and Thua-nao were pointed onto LB agar containing Pb(II) at a concentration of 200 mg/L and Cd(II) at concentration of 10 mg/L, then the plates were incubated at 37°C for 24 h. Mucoid colonies were randomly selected and re-streaked onto a fresh nutrient agar plate (NA) until purified colonies were obtained. All isolated were identified according to morphological and biochemical properties (Burgey's manual of systematic 2^{nd}, 2009). Further, the the selected strains were identify the 16S rRNA gene and compared to 16S rRNA sequence a public database using in BlastN program. The nucleotide sequence coding for 16S rRNA gene has been submitted to GenBank database.

The inoculums were prepared by growing Pb(II) tolerance isolates in LB broth pH7.0 at 30°C and shaking for 15 h. Cells were pelleted by centrifugation at 5,000 rpm, 4°C for 15 min and washed twice sterile 0.85% normal saline and then re-suspended in sterile normal saline solution to obtain OD550= 0.5. Ten percent of inoculums (v/v) were transferred into 50 ml of glucose salt medium broth containing 0, 100, 300, 500 and 700 mg/L of Pb(II) and 0, 5, 10, 15, 20 mg/L of Cd(II) concentration then incubated at 30°C for 24 h with shaking at 120 rpm. After that bacterial suspensions were serially diluted and spread on NA (Hamzah *et al*., 2009).

4. Purification of γ-PGA

γ-PGA was purified by the modified method of Goto and Kunioka (1992). Cells were separated from culture broth by centrifugation at 10,000 rpm, 4°C for 20 min. The supernatant containing γ-PGA was poured into four volumes of cold ethanol and kept for 12 h at 4°C. Crude γ-PGA was collected by centrifugation at 10,000 rpm, at 4°C for 30 min, then the aqueous γ-PGA solution was desalted by dialysis against distill water for 12 h at 4°C and lyophilized to keep pure γ-PGA.

5. Characterization of γ-PGA

γ-PGA samples were hydrolyzed in 6 M HCl at 110°C for 24 hr. The solution of hydrolyzed γ-PGA was adjusted pH to 7.0. The amino acids were determined with Thin-layer chromatography. Samples of PGA solution were performed on silica gel plate with solvent systems of butanol-acetic acid-water (3:1:1, w/w) and 96% ethanol-water (63:37, w/w). Amino acids were detected by spraying with 0.2% ninhydrin in acetone.

The ratio of L-, D- glutamic acid monomer unit in γ-PGA was measured by High Performance Liquid Chromatography (HPLC) using ACE-HPLC (C18) column and acetonitrile : H_2O solution as eluent at flow rate of 1.0 ml/min with UV detector was used.

6. Determination of heavy metals sorption by γ-PGA and bacterial cells

6.1 Optimum pH for adsorption

Stock solution (1,000 mg/L) of cadmium(II)chloride (CdCl2) and (5,000 mg/L) of lead (II) nitrate (Pb(NO3)2) were prepared in deionized water. In vitro binding experiments, with 100 mg/L of Pb(II) and 20 mg/L of Cd(II) were carried out by metal ion stock solution in 50 ml volumetric flasks and adjusting to pH 3, 4, 5, 6, and pH 7. Next, 10 mg/L of γ-PGA was added and the mixture was shaken at 120 rpm, at room temperature for 2 h. The solution samples were taken at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 and 120 min. Then filtered through 0.2-μm membrane filter and the filtrate were analyzed for residual heavy metal concentration.

6.2 Optimum temperature for adsorption

The effect of variation of temperature on γ-PGA adsorption was studied at different temperature at optimum pH. Stock solutions of heavy metals were obtained to working solution of desired concentration. *In vitro* binding experiments, with 100 mg/L of Pb(II) and 20 mg/L of Cd(II) carried out by taking metal ion stock solution in 50 ml volumetric flasks and adjusting to temperature at 25, 35 and 45°C. Next, 10 mg/L of γ-PGA was added and the mixture was shaken at 120 rpm, at room for 2 h. The solution samples were taken at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 and 120 min. Then filtered through 0.2-μm membrane filter and the filtrate were analyzed for residual heavy metal concentration.

6.3 Study of initial Pb(II) concentration

Study of Pb(II) binding isotherm studies was prepared by taking Pb(II) concentrations at 75, 100, 250, 500 and 700 mg/L. For Cd(II) isotherm studied, the experiments concentrations varied at 5, 10, 20, 30 and 50 mg/L, adjusted each to pH 5 and added with 0.01 mg of γ-PGA separately at 35°C for 2 h. Samples were taken at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 and 120 min. Then filtered through 0.2-μm membrane filter and the filtrate were analyzed for residual heavy metal concentration.

6.4 Stability of γ-PGA

Stock solution (1,000 mg/L) of γ-PGA was kept at 30 and 40 °C for 1, 3, 5 and 7 days. Then *in vitro* binding experiments were carried out by taking 10 ml of γ-PGA solution in volumetric flask and adjusting to optimum pH (pH5). Next, 100 and

20 mg/L of Pb(II) and Cd(II), respectively and the mixture was shaken at 120 rpm, at room for 2 h. The solution samples were taken at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 and 120 min. Then filtered through 0.2-µm membrane filter and the filtrate were analyzed for residual heavy metal concentration.

7. Determination of heavy metals sorption by bacterial cells

7.1 Preparation of the dried powdered cells

The tolerance bacterial cells were cultured in LB broth at 37°C, pH 7.5 with shaking at 120 rpm for 12 h. Bacterial cells were harvested from growth medium by centrifugation at 5,000 rpm for 15 min and cell pellet washed twice with 0.2 M phosphate buffer pH 7.2 and dried in an oven at 70°C for 24 h. The dried cells were then grinded in a mortar and pestle, stored in zip lock bag and kept in a desiccator until used.

7.2 Heavy metals adsorption studies

In vitro binding experiment was carried out by taking 1 ml of 5,000 mg/L of Pb (II) solution and 1 ml of 1000 mg/L of Cd(II) solution adjusting to desired pH then the volume was adjusted to 50 ml in 250-ml Erlenmeyer flask. Next dried bacterial cells were added and the reaction mixture was shaken at 120 rpm for 2 h. Samples were taken at time intervals then centrifuge at 12,000 rpm for 5 min. Supernatant was analyzed for residual Pb(II) and Cd(II). Dried cell concentrations were varied from 0.25-5 g/L. Optimum pH for Pb(II) sorption was performed at pH 3, 4, 5, 6 and 7 at 30°C. Effect of temperature on Pb(II) and Cd(II) adsorption were done at 25, 35 and 45°C, pH 5.0 with initial of Pb(II) at 100 mg/L and 20 mg/L for Cd(II).

The isotherm experiments were studied using as 0.1 g of cell dried. Biosorbent was suspended in 50 ml of metal solution and initial Pb(II) concentration were varied over the range 50-700 mg/L. For Cd(II) experiments, an initial concentration of Cd(II) solutions were varied at 10, 20, 30 and 50 mg/L. The working solution adjusted to pH 5 and the temperatures of experiment were shaken at 100 rpm for 2 h. at 35°C. Samples were taken at time intervals, centrifuged and then analyzed for residual metal concentration

8. Determination of heavy metals on biosorbents

Heavy metals remaining in solutions were analyzed by using a flame atomic absorption spectrophotometer (AAS) (Varian SpectrAA FS-220 model) with an oxidizing flame of an air-acetylene mixture at a ratio of air flow of 13.50 L/min and acetylene flow 2.00 L/min.

9. Data evaluations

The amount of adsorbed by γ-PGA and biomass cell were calculated from the difference between the metals quantity added to the biosorbent and the metal content of the supernatant using the following equation (1). Langmiur and Freundrich isotherms were employed to analyze the biosorption data and the methods were described as follows equations (2), (3). The deviation of theoretical isotherm models from the experimental data was measured using coefficient of determination (R^2) from Microsoft Excel function. Moreover, the effect of isotherm shape can be used to predict whether a sorption system is defined by the following relationship equation (4).

RESULT AND DISCUSSION

1. Isolation of γ-PGA producing bacterial strains from fermented soybean and soil.

In this study, *Bacillus* strains were isolated from fermented soybean in Thailand and from soil collected in Maesot district Tak province. Appendix Figture1 and 2 (Appendix C) illustrated that the isolated bacteria produce γ-PGA by SDS-PAGE method and was used γ-PGA from *B. subtilis* NBRC16449 as standard. In this screening, 78 isolates of γ-PGA producing strains were obtained from fermented soybean and 20 isolates were obtained from soil. The morphological and physiological characteristics of the PGA producing isolates were rod-shaped, spore-forming, gram-positive, thermo tolerant bacteria. In addition, the isolates showed catalase activity, described in Table Appendix B.

Previously, Thua nao were isolated and characterized by several researchers. It has been associated genus *Bacillus* species as predominant microorganism in these fermented soybeans. Similar fermented soybean products in several countries such as, Indian kinema, Chinese douche, Japanese natto, Korean chunkukjang, Korean meju and Nigerian daddawa were screened and isolated and identified as *Bacillus* strains (Chantawannakul *et al*., 2002; Inatsu *et al*., 2002; Mo *et al*., 2010). Furthermore, it could be reported other bacterial species such as *Lactobacillus* sp., *Enterococcus* species and yeast were also detected (Sakar *et al*., 1994; Chukeatirote *et al*., 2006). Besides, γ-PGA producing strains were isolated from fermented soybean products. *Bacillus* species were isolated as PGA-producing bacteria from soil and water (Hoppensack *et al*., 2003; Xu *et al*., 2005; Mahmoud, 2006; Meerak *et al*., 2007).

2. Isolation and characterization of metals tolerant bacteria

126 isolates of Pb(II) and Cd(II) tolerant bacteria were isolated and six isolates designated as LP126, CR002, PR001, PY004, MS104 and MS406 grew well in the glucose salt medium contained 700 mg/L of Pb(II) and 20 mg/L of Cd(II). All of them were identified as genus *Bacillus*.

2.1 Lead tolerant bacteria

Six isolated strains exhibited Pb(II) characteristic at high concentration of Pb(II). However, the growth decreased when the concentration of Pb(II) added into growth medium was increased (Fig. 4). The isolate CR002 was the most tolerant species toward Pb(II) since the reduction in growth was only 35% at 700 mg/L Pb(II) after 24 h. Harithsa *et al*. (2002) indicated sulfate-reducing bacteria isolated from salt pans were grown better than control in the presence of 500 μg/ml Pb(II). In the studies on biosorption of Pb(IV) by *B. subtilis* was show the maximum bacterial growth as 4.90×10^8 cell/ml for 700 mg/L Pb(IV) concentration (Masud Hossain and Anantharaman, 2006). Valusamy *et al*. (2011) demonstrated that the growth of *B. megaterium* X4 isolated from hydrocarbon contaminated soil was multiple metal resistance to Cu(II), Pb(II) and Cd(II) at concentrations of 50, 50 and 10 mg/L, respectively. According, *P. aeruginosa* ASU 6a was isolated from metal-contaminated water of mining that showed highly resistant to 500 mg/L of Pb(II) and Pb(II) biosorption capacities by a tolerant bacterial strain was also investigated (Gabr *et al*., 2008).

Although, few studies have investigated the correlation between metal bioavailability and microbial tolerance, in apparent that isolate CR002 was tolerate to different high levels of Pb(II) stress. Comparison which another studies, it was clearly The isolate CR002 was considerably better than those reported for other bacteria (Harithsa *et al*. 2002; Gabr *et al*., 2008; Valusamy *et al*. 2011)

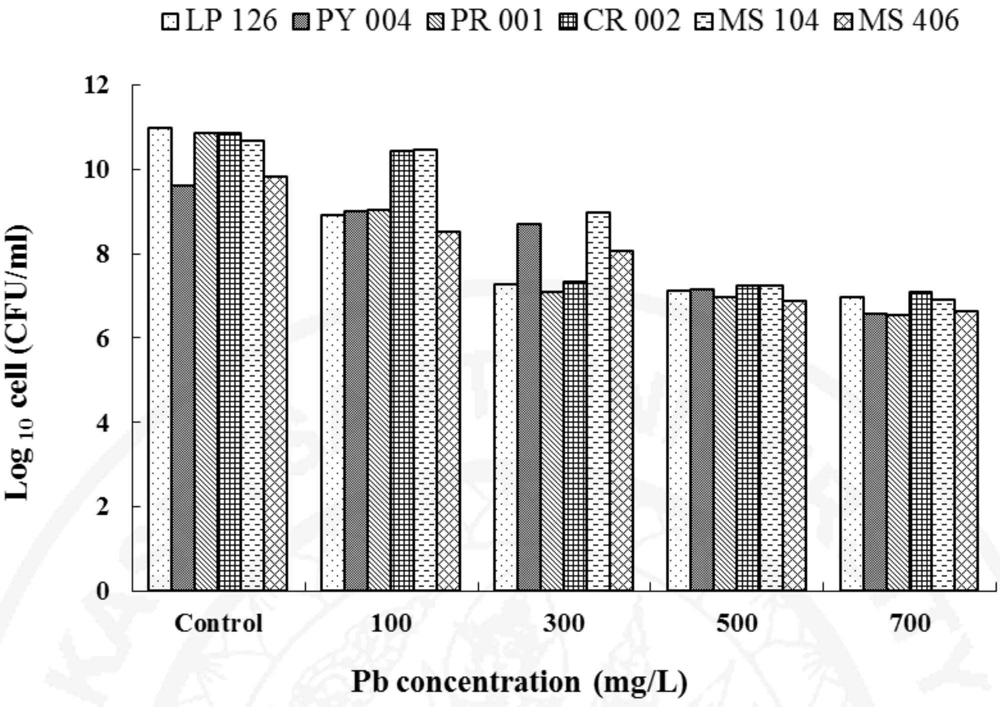


Figure 4 Cell viability in glucose salt medium with different concentrations of lead

Effect of metals to bacterial growth, morphological and bacterial activities have been reported. Biosorption of lead by *Phanerochaete chrysogenum* biomass was strongly affected by pH (Niu *et al*., 1993). Roane (1999) found that *B. megaterium* and *P. marginalis* had different of Pb tolerance mechanism. *B. megaterium* showed intracellular sequestration of lead in their cells, but *P. marginalis* produced an extracellular. Furthermore, the isolate CR002 also produces γ-PGA assuming that the polymer may play an important role in Pb tolerant and adsorption (Table 3). *Bacillus* cells were capable to uptake heavy metal and proposed that carboxyl group, associated to the peptidoglycan layers of the cell wall significantly in the uptake of heavy metals mechanism (Carlos *et al*., 2001). An extracellular polymers secreted by bacteria, fungi and algae play an important role in heavy metal removal because of their extensive capacity including carboxyl, phosphonate, amine and hydroxyl groups (Vegliò *et al*., 1997; Salehhizadeh and Shojaosadati, 2003; Kang *et al*., 2007).

2.2 Cadmium tolerant bacteria

Cadmium tolerant strains were detected in glucose minimal salt medium supplemented with 5 to 20 mg/L of Cd(II). The six bacteria strains exhibited different growth patterns in the presence of various cadmium concentrations (Fig. 5). Cd(II) resistant strain grew in the presence 20 mg/L was *B. subtilis* MS104 showed the most resistance to cadmium. The tolerant isolate were decreased 56% cell viability compared with control.

In comparison to previous studies on the Cd(II) tolerance bacteria strains. It has been showed six copper-resistant bacterial strains were isolated from wastewater and identified as genus *Sallmonella*, *Streptococcus pyrogenes*, *Vagococcus fluvalis* and *E. coli*. All strains showed resistance toward Cd(II) in LB broth containing 250-350 mg/L of Cd(II) (Shakoori and Muneer, 2002). Seven bacterial strains were isolated from oil sludge pond and could be tolerant to 100 mg/kg of Cu(II) and Cd(II). They also adsorbed Cd(II) into the growth medium and isolate B21 was the most tolerant which 29% reduction compared with control (Hamzah *et al*., 2009). In another study, reported by *B. thuringienesis* was carried out in minimal medium with supplement 0 to 8 mg/L of Cd(II) and showed a long lag phase in the presence Cd(II) (Rathnayake *et al*., 2009). Guo *et al*. (2010) showed that *Bacillus* sp. was isolated from surface a cadmium hyperaccumulate plant. They also reported that the strain had been a higher sensitivity to Cd(II) with 23.86% growth inhibition observed at 100 mg/L of Cd(II) and the lag phase was extended.

Cadmium is more established in the environment for a long time thus microorganism can be adapted mechanism toward Cd toxicity (Vig *et al*., 2002). Silver *et al* (1989) stated that the plasmid codes a Cd(II) efflux transport system which rapidly transport Cd(II) out of bacteria cell. The *cadA* operon has been reported to provide cadmium resistance in *B. subtilis, Staphylococcus aureus, Pseudomonas putida* (Nucifora *et al*., 1989; Tsai *et al*., 1992; Lee *et al*., 2001). Furthermore, it has been suggest that metal-binding proteins is mediated by mellothionine- encoding gene witch synthesized under heavy metal stress conditions in prokaryotes and eukaryotes such as yeast (Strouhal *et al*., 2003).

In a part of this study, the results showed the potential applicability of the recently isolated tolerant strains. The efficient of *Bacillus* sp. CR002 was growth over a range of Pb(II) concentrations under the stress conditions. It may be represented an advantageous aspected for metal remediation. While in our study, the levels of Cd(II) tolerance showed by strain *B. subtilis* MS104 was extensively Cd(II) lower than that reported in the literature due to the source of isolated strain was low level of Cd. Further we would be improved or modified of the binding sites on *B. subtilis* MS104 to enhance the biosorption capacity.

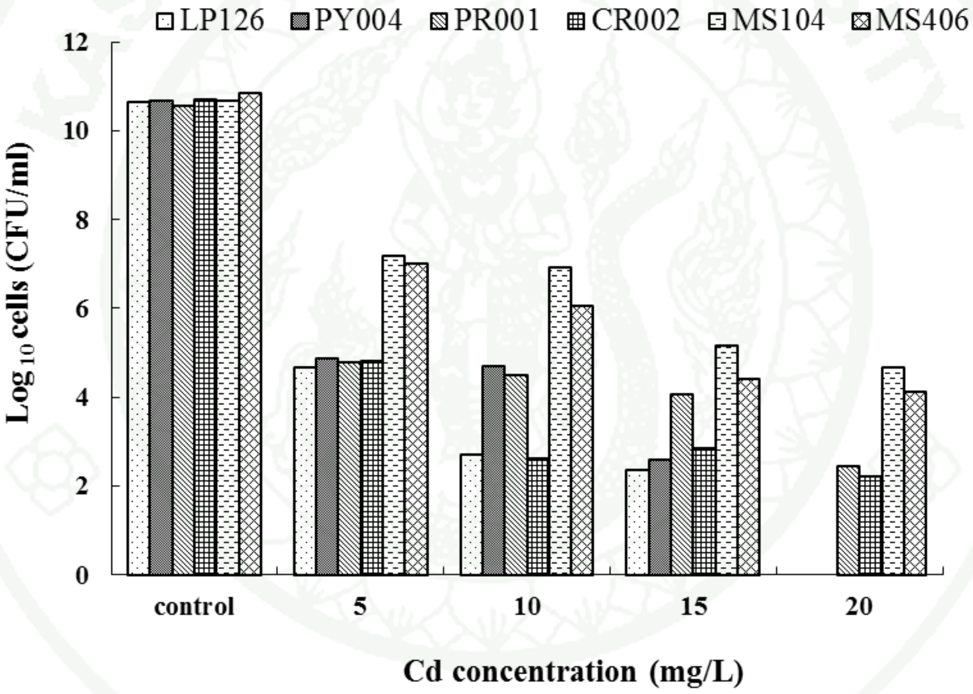


Figure 5 Cell viability in glucose salt medium containing cadmium at 5-20 mg/L

3. γ-PGA production of isolated bacterial strains

Bacteria isolated from soil and Thai fermented soybean were capable of producing γ-PGA when grown aerobically in a PGA production medium containing 1% ammonium sulfate and 2% glucose as sources of nitrogen and carbon, respectively. In preliminary, γ-PGA produced were tested with SDS-PAGE method and then collected 36 isolates test for amount of γ-PGA per liter. The experiment showed that *B. subtilis* NT147 could produce a large amount of γ-PGA about 8.14 g/L (Table 3).

It has been assumed that PGA highly molecular weight such as depending on bacterial strains used, broth components and culture conditions (Park *et al*., 2005). Several on the production of γ-PGA were conducted by researchers. The maximum of γ-PGA production 8.1 g/L was obtained from *B. lichenisformis* A35 growing in M medium without L-glutamic acid (Cheng *et al*., 1989). On 1992 Kunioka and his co-worker found that *B. subtilis* IFO 3335 could produce γ-PGA about 9.6 g/L in medium containing L-glutamic acid , citric acid and ammonium sulfate. Recently, the studied of solid substrate fermentation (SSF) using as the basis dairy manure, soybean meal, wheat bran and swine manure have been used for γ-PGA production. The first studied on optimization γ-PGA production in SSF was obtained good mix substrates (Xu *et al*., 2005). Chen *et al* (2005) investigated and optimized of γ-PGA production in swine manure and dairy manure but found lower yields of γ-PGA (4.5% w/w). However, previous reports on production of γ-PGA in SSF were very poor. On 2008, the researchers were optimized solid substrates. They found that soybean meal have been the best substrate for γ-PGA production and gave maximum yield of 99 mg/gds^{-1} of γ-PGA by *B. licheniformis* NCIM2324 (Bajaj *et al*., 2008). Not only carbon sources and nitrogen sources but also factors such as medium pH, temperature, aeration and ionic strength have been effect on productivity and quality of γ-PGA (Shih and Van, 2001).

Table 3 Production of γ -PGA by the isolated bacteria

Isolate	γ-PGA(g/L)	Isolate	γ-PGA(g/L)
LP119	0.414	NT145	1.562
LP120	0.314	NT146	1.523
LP124	0.482	NT147	8.140
LP125	0.311	NT148	1.352
LP126	0.254	NT149	1.106
LP127	0.284	NT150	2.020
LP128	0.800	CR002	1.48
LP129	0.300	CR026	2.09
LP130	0.934	PR001	1.08
LP131	0.596	PR003	1.35
LP132	0.175	PY003	0.80
LP133	0.336	MS303	1.22
LP134	3.060	MS403	2.045
LP136	0.846	MS404	0.99
LP137	0.540	MS506	1.93
LP139	2.310	MS511	3.01
NT141	1.156	*B. subtilis*	8.00
NT142	2.016	NBRC16449	
NT 143	7.020		
NT 144	1.260		

4. Analysis of hydrolyzed products

PGA samples (2mg/ml) were completely hydrolyzed by 6M HCl at 110°C for 24 h. PGA samples were characterized by amino acid and thin-layer chromatography (TLC). TLC of the hydrolysate performed on a cellulose thin-layer plate and visualized with 0.2 % ninhydrin indicated single spot with a R_f value identified to the authentic glutamic acid. The monomer unit of PGA produced as shown in Table 4, *Bacillus* sp. CR002 was composed of both D-glutamic acid and L-glutamic in a ratio 61.4:38.6. Whereas, *B. subtilis* NT147 was rich in the D-isomer of glutamic acid that consisted of D- and L- glutamic acid in a ratio of 61.1:38.9.

Table 4 Ratio of D-glutamic acid to L-glutamic acid in PGA produced by *B. subtilis* NBRC16449, *B. subtilis* NT147 and *B. subtilis* CR002

polymer	Composition of glutamic acid isomer (%)	
	D-Glu	L-Glu
Bacillus sp. CR002	61.4	38.6
B. subtilis NT147	61.1	38.9
B. subtilis [a] NBRC16449	70	30

Source: [a] Meerak *et al*. (2007)

5. Biosorption of heavy metals by γ-PGA

5.1 Effect of pH on biosorption capacity

In previous study have shown that pH is significant factor on heavy metals adsorption process. In this study, biosorption of Pb(II) and Cd(II) as a function of pH measured, and the result are shown on Fig. 6. The experiments were adjusted different initial pH values from 3.0 to 7.0. Fig. 6 illustrates the effect of pH on Pb(II) and Cd(II) adsorption by γ-PGA of *Bacillus subtilis* NBRC16449 and *B. subtilis* NT147. With a rise in pH from 3.0 to 7.0 for Pb(II) solution, the amount of lead removal by γ-PGA were increased 52%, 57%, 61%, 72% and 74% for γ-PGA of *B. subtilis* NT147. Similarly, the adsorption on Pb(II) by γ-PGA of *B. subtilis* NBRC16449 showed percentage Pb(II) removal 48%, 51%, 50%, 57% and 61%, respectively. In addition, at higher pH value lead precipitation was dominant and aqueous metal hydroxide formation may be important mechanisms in the adsorption process (Kumar *et al*., 2010).

The percentage removal of cadmium by γ-PGA increased as the pH of solution increased and reached a maximum value at pH 5 for γ-PGA of *B. subtilis* NT147 and pH 6 for γ-PGA of *B. subtilis* NBRC16449. There was shown the percentage for remove Cd(II) in solution as 17%, 31%, 32%, 30% and 25% for γ-PGA of *B. subtilis* NT147 and 16%, 25%, 25%, 29% and 20% for γ-PGA of *B. subtilis* NBRC16449.

Previous study on heavy metal biosorption , the pH of solution system was the most important parameter affect to solubility of metals ion and the activity of functional groups of the biosorbent for example carboxylate, phosphate and amino groups. According to pH below the pK_a value of γ-PGA (4.09), the carboxyl group is plays a major in role in γ-PGA structure primarily in the protonated form to prevent for the metal sorption. In addition, the tendency of γ-PGA could be to form intramolecular hydrogen bond at low pH with more compact α-helx conformation (Shih and Van, 2001; Ho *et al*., 2006; Inbaraj *et al*., 2009).

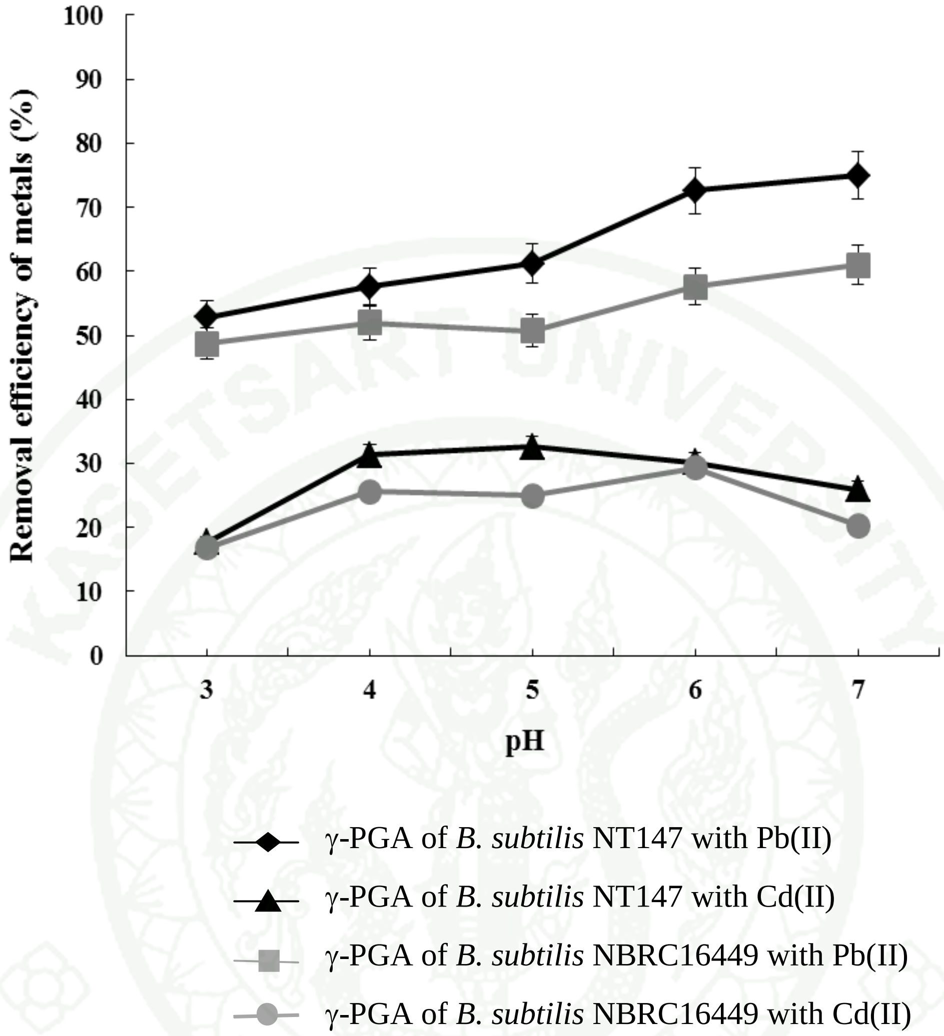


Figure 6 Effect of solution pH on Lead(II) and Cd(II) adsorption by γ-PGA (C_0: 100 mg/L for Pb(II); 20 mg/L for Cd(II), γ-PGA: 10 mg/L, 100 rpm, 30°C)

Siao *et al* (2009) have been reported that at pH>2.5 for lead and pH>3 for cadmium were presumably the carboxyl groups with a concurrent conformational change from α-helix to random coil. However, at pH< pK_a the metal adsorption did occur at pH 3 to 4, causing of the α-helix structure of γ-PGA to generate a electrostatics field to facilitate metal binding at pH< pK_a. In addition, there is also difference variation in the adsorption capacity between Pb(II) and Cd(II), as shown in Fig 6. It is probable that the difference in electronegativity (2.33 for lead and 1.69 for cadmium) causes in difference in binding capacity.

5.2 Effect of temperature on biosorption

Another factor that affects biosorption is the temperature. The effect of temperature may enhance bisorption due to the increased surface activity and kinetic energy of the solute (Sag and Kutsal, 2000; Vijayaragvan and Yun, 2007). Increasing of temperature will increase the capacity of the adsorbent diffusion across surface layer or internal pores of adsorbent particles as liquid viscosity decreases whereas temperature increases. Another effect of temperature on biosorption is the equilibrium capacity of adsorbent which relies on the adsorption process is consisting of exothermic or endothermic reaction (Al-Qodah, 2006).

The effect of temperature on Pb(II) and Cd(II) biosorption by γ-PGA. Experiments were performed at temperatures of 25°C, 35°C and 45°C for adsorption process. The result of Pb(II) adsorption experiments are depicted in Fig. 7. For γ-PGA of *B. subtilis* NT147, the experiments indicate that an important change in the extent of adsorption with increase temperature. The γ-PGA of *B. subtilis* NBRC16449 also showed temperature effect on adsorption for 45°C. For instance, at temperature of 45°C showed that maximum removal of total Pb in experiment system 95% by γ-PGA of *B. subtilis* NT147, whereas 72% of the total Pb(II) removed at 35°C by γ-PGA of *B. subtilis* NBRC16449. In addition, as the temperature increases, the adsorption capacity of Cd(II) is not significant varied in γ-PGA of *B. subtilis* NT147 adsorption experiments. The percentage of removal on Cd(II) biosorption by γ-PGA of *B. subtilis* NT147 was 32% at 45°C. Besides, the adsorption of Cd(II) on γ-PGA of *B. subtilis* NBRC16449 was found to be 25% of Cd(II) removal at 35°C as indicated in Fig. 7.

Several researchers have also investigated the effect of temperature on biosorption of heavy metals by using different biomass and found similar results with this study. In a study performed by Aksu *et al* (2008) also investigated the effect of temperature on biosorption of dye by chitosan and their results indicated that temperature at 45°C with further increased surface activity.

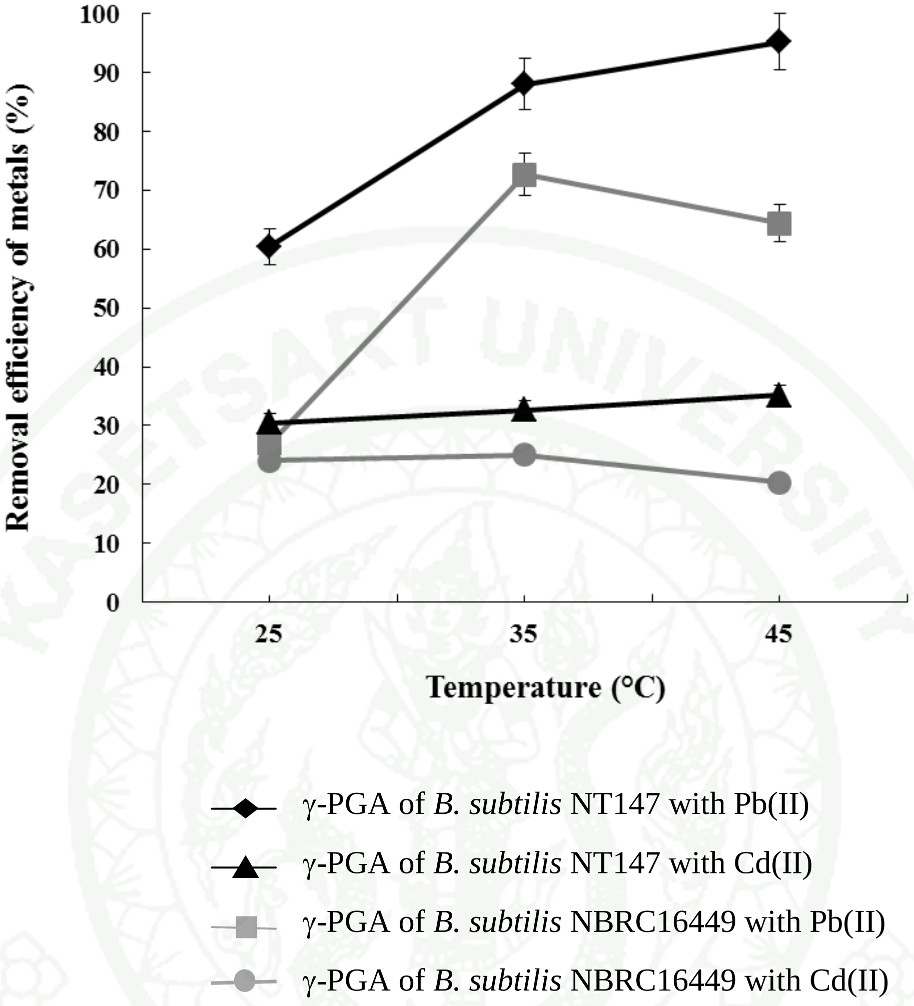


Figure 7 Effect of temperature on Pb(II) and Cd(II) adsorption by adsorption by γ-PGA (C_0: 100 mg/L for Pb(II); 20 mg/L for Cd(II), pH 5, γ-PGA: 10 mg/L, 100 rpm)

Although, the influence of pH and temperature on Pb(II), Cd(II) adsorption by two different γ-PGA. The comparison removal efficiency metals, it showed that percentage removal metals of γ-PGA of *B. subtilis* NT147 better than γ-PGA of *B. subtilis* NBRC16449. Because of the properties of γ-PGA that may be a drawback to biosorption, however, more research will be necessary.

5.3 Effect of initial metal ion concentration

Heavy metal concentration also affects the efficiency of metal removal. The initial concentration seems to influence on the adsorption. This is because initial concentration provides impact driving force between the solute and solid phase (Aksu, 2005).

The results in Table 5 revealed that specific metal uptake increased with increasing initial Pb(II) concentration from 75 to 700 mg/L on adsorption process. These results indicated that Pb(II) uptake by γ-PGA was more efficient in biosorption at different bacteria strains produced. For γ-PGA of *B. subtilis* NT147 the efficiency values decrease from 85.45 to 21.43 %. Pb(II) at concentration of 66 mg/L showed more than 80 % removal efficiency and accumulated lead onto γ-PGA 564 was mg metal/g γ-PGA. At the same concentration of Pb(II) concentration the efficiency values decrease from 87.9 to 16.1% for γ-PGA of *B. subtilis* NBRC16449. The maximum Pb(II) removal percentage was 87.9% and uptake was 564.7 mg metal/g γ-PGA at initial Pb(II) concentration of 70 mg/L.

In this studies presence the effect of initial cadmium concentration in the range of 5 to 50 mg/L on adsorption experiments as shown in Table 5. The uptake capacity has increased from 13.2 to 81 mg metal/g γ-PGA and the percentage of cadmium removal was decreased from 30% to 21% for γ-PGA of *B. subtilis* NT147. Whereas Cd(II) uptake by γ-PGA of *B. subtilis* NBRC16449 has increased from 10.2 to 49.1 mg metal/g γ-PGA and the percentage of cadmium removal was decreased from 25% to 9%, respectively. However, the increases of uptake capacity of biosobent with increase metal concentration ion is reasonable due to higher interaction between metal ions and adsorbent (Şahin and Ö ztürk, 2005). From table 5 and 6 there are clearly that higher adsorption efficiency were observed at lower concentrations of metal ion for each γ-PGA.

Previous reports by several researcher that increasing initial metal concentrations in solution resulted in increasing its adsorption efficiency until saturation on adsorbent sites (Green-Ruiz, 2006). The first, most important one is that adsorption sites remain unsaturated during the adsorption reaction. The second cause is the adsorbent mass on the biosorption of various metal ions aggregation/ agglomeration of adsorbent particles higher concentrations (Yasemin and Tez, 2007).

Table 5 Biosorption of different concentrations of lead by γ-PGA of *B. subtilis* NT147 and γ-PGA of *B. subtilis* NBRC16449 at pH 5, γ-PGA: 10 mg/L, 100 rpm, temperature 35°C

Biosorbent	Initial lead conc. (mg/L)	Residual lead conc. (mg/L)	% Removal of lead	Specific lead uptake (mg/g γ-PGA)
γ-PGA of *B. subtilis* NT147	66.094	11.658	82.36	54.437
	84.670	17.155	79.73	67.514
	235.256	150.160	36.171	85.096
	408.653	305.929	25.137	102.724
	705.128	577.243	18.136	127.724
γ-PGA of *B. subtilis* NBRC16449	70.059	9.437	86.528	60.621
	93.821	20.350	80.112	81.974
	243.589	157.371	35.394	86.217
	411.858	330.769	17.769	95.382
	703.205	593.75	15.565	109.455

Table 6 Biosorption of different concentrations of cadmium by γ-PGA of *B. subtilis* NT147 and γ-PGA of *B. subtilis* NBRC16449 at pH 5γ-PGA: 10 mg/L, 100 rpm, temperature 35°C

Biosorbent	Initial cadmium conc. (mg/L)	Residual cadmium conc. (mg/L)	% Removal cadmium	Specific cadmium uptake(mg/g γ-PGA)
γ-PGA of *B. subtilis* NT147	4.469	3.143	29.675	1.326
	7.592	5.285	30.381	2.306
	20.676	13.927	32.641	6.749
	26.529	19.222	27.541	6.500
	37.575	29.468	21.573	8.106
γ-PGA of *B. subtilis* NBRC16449	4.202	3.173	24.479	1.028
	7.936	6.193	21.969	1.743
	20.6391	15.477	25.009	5.161
	26.021	19.911	23.478	6.109
	49.653	44.734	9.906	5.612

5.4 Adsorption isotherms

The capacity of the adsorbent can be elucidated by equilibrium sorption isotherm. The adsorption isotherm is a graphical representation the relationship between the liquid phase and the solid phase when the adsorption process reaches an equilibrium state. The analysis of the isotherm data by fitting them was significant step to finding a suitable model (Deng *et al*., 2007; Kumar *et al*., 2010) The Langmiur (Eq. (2)) and Freundrich (Eq. (3)) models are generally used models in the case of adsorption of metals ions by adsorbents to estimated which best fits the experimental data.

Adsorption equilibrium of Pb(II) by γ-PGA at different initial concentrations were well described by Langmiur and Freudrich isotherm models, as shown in Fig. 8, 9. From Table 6, the Freundrich model yielded reasonably good fit to the experimental data for two different γ-PGA when observed on the highest R^2-value (0.947, 0.946) more than Langmiur isotherms. The values of the constants K_F and 1/n were calculated to be 36.057 and 0.188 for γ-PGA of isolate NT147, 51.238 and 0.112, respectively for isolate NBRC16449. Since the value of 1/n is less than 1, it indicates a favorable adsorption.

Whereas, it can be seen from Table 7 that the isotherm data fits the Langmiur equation more poorly (R^2= 0.911, 0.900) than the Freundlich equation. The maximum adsorption capacity (Q_0) of the γ-PGA of isolates NT147 and NBRC16449 obtained from the Langmuir model was 107.526, 100 (mg/g), respectively. The binding constant *b* value was essential features of Langmuir isotherm can be expressed in terms of a dimensionless constant separation factor (R_L) that is given as follow equation (4). The values of R_L were 0.153 and 0.077 for γ-PGA of isolates NT147 and NBRC16449 that indicated favorable adsorption in the process.

In addition, the adsorption Cd(II) by γ-PGA, Fig. 10 and 11 illustrate that the biosorption data were fitted to the two adsorption isotherms of Langmuir and Freundlich. The adsorption isotherm constants of Cd(II) with the corresponding correlation coefficients (R^2) and the Langmiur model yielded a good fit to the experimental data with both the highest R^2-value of 0.991 and 0.981 for γ-PGA of *B. subtilis* N T147 and γ-PGA of *B. subtilis* NBRC16449, respectively. The maximum capacity q_m values predicted by the Langmiur model were 46.082 mg/g (γ-PGA of *B. subtilis* NT147) and 18.115 mg/g (γ-PGA of *B. subtilis* NBRC1644). The values of R_L were 0.242 and 0.942 for γ-PGA of isolates NT147 and NBRC16449 that indicated favorable adsorption in the γ-PGA of isolates NT147 process but it was poorly as γ-PGA of isolates NBRC16449. The adsorption of Cu(II) ions γ-PGA was reported the adsorpt ion capacity by Langmuir isotherm with Q_0 = 1.73, 43.5 and 77.9 mg/g, respectively (Mark *et al*., 2006).

On the other hand, it has been investigated the biosorption system of Pb(II) and Cd(II) on γ-PGA was reported to also be well modelled by the Toth isotherm (Siao *et al*., 2009). The adsorption capacity of the γ-PGA in this study was higher than other biosorbent mentioned and a smaller value of *b* indicated that the adsorbent had a higher binding affinity for the biosorbent. Therefore, also it can be indicated that the γ-PGA in this study has the great affinity for Pb(II) and Cd(II).

Table 7 The Langmiur and Freundrich sorption constants for biosorption of Pb(II) and Cd(II) bγ-PGA at different initial concentrations

		Lanngmiur constants				Freundrich constants		
Adsorbent	metal	q_m (mg/g)	*b*(L/mg)	R^2	R_L	K_F	*n*	R^2
γ-PGA NT147	Pb	107.526	0.089	0.911	0.153	36.057	5.312	0.947
	Cd	46.082	0.009	0.991	0.242	1.779	1.193	0.954
γ-PGA NBRC16449	Pb	100.00	0.171	0.900	0.077	51.238	8.865	0.946
	Cd	18.115	0.018	0.981	0.929	1.976	1.363	0.865

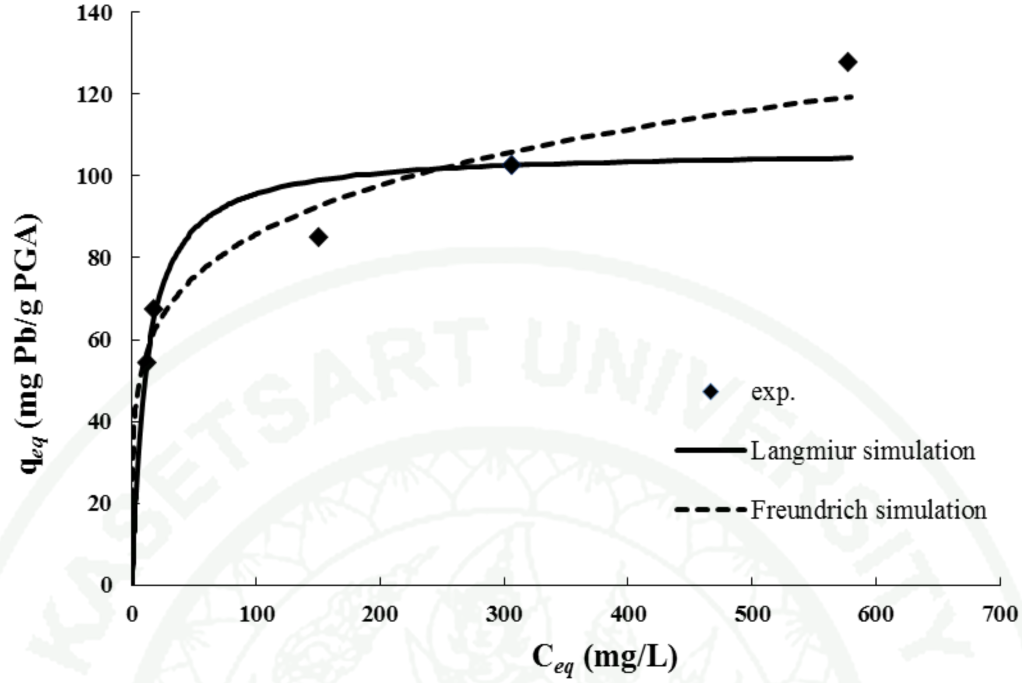


Figure 8 Adsorption isotherms of Pb(II) by γ-PGA of *B. subtilis* NT147

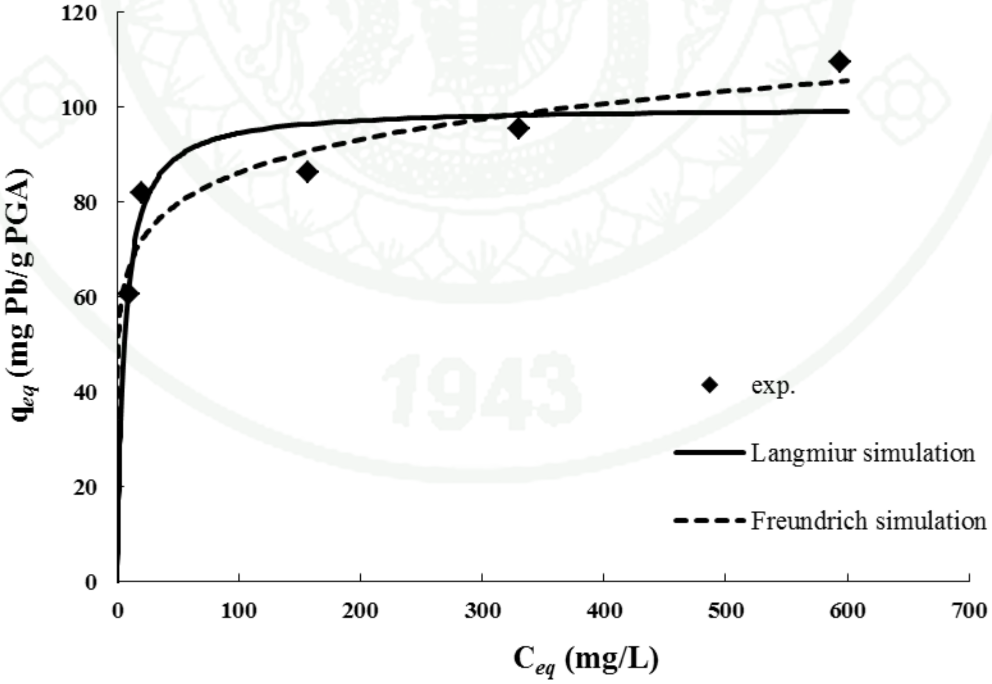


Figure 9 Adsorption isotherms of Pb(II) by γ-PGA of *B. subtilis*

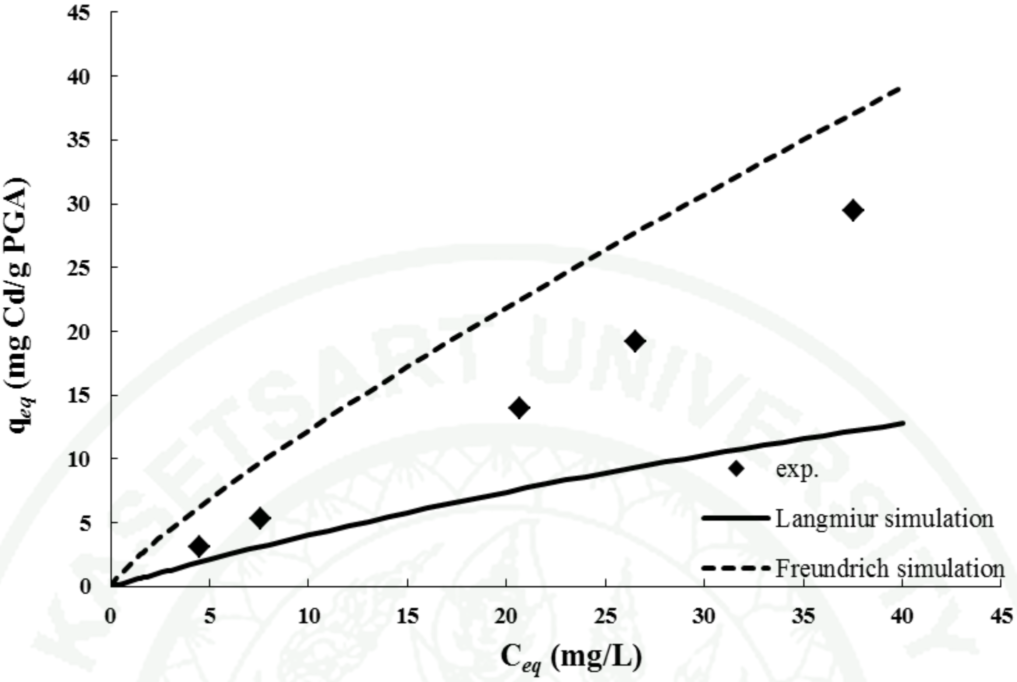


Figure 10 Adsorption isotherms of Cd(II) by γ-PGA of *B. subtilis* NT147

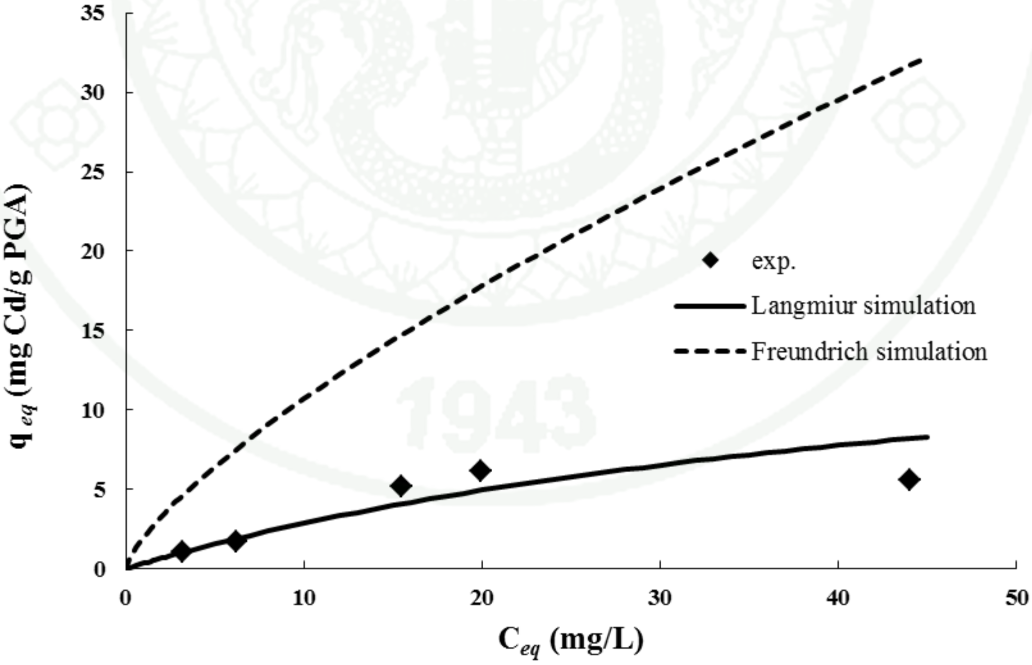


Figure 11 Adsorption isotherms of Cd(II) by γ-PGA of *B. subtilis* NBRC16449

5.5 Effect of temperature on stability of γ-PGA

Stabilities of γ-PGA against difference temperature were study. The result revealed that the Pb(II) and Cd(II) adsorption gradually declined after one day when γ-PGA was incubated at 40°C. The amount of Pb(II) adsorbed nearly decreased to 6, 16 and 25% for γ-PGA of *B. subtilis* NBRC16449 and 3, 6 and 19% for γ-PGA of *B. subtilis* NT147 at the same concentration and temperature(Fig. 12(a) and 13(a)). Whereas, the capacity of Cd(II) adsorption were significantly decreased at two different temperature. The relative activity on Cd(II) adsorption were decreased about 50% after seven days stored of γ-PGA of *B. subtilis* NBRC16449 in incubator at 40°C and 25% for γ-PGA of *B. subtilis* NT147 (Fig. 12(b), 13(b)).

Goto and Kunioka (1992) have been reported that solution of γ-PGA was hardly hydrolyzed for 1h of heating at 80°C. However, it was hydrolyzed readily by heating at 120°C. An activation energy approximately 120 kJ/M was used randomly for hydrolysis of γ-PGA. The relative bond strengths of C-C, C-N and C-O bonds are 347 kJ/M, 305 kJ/M and 359 kJ/M, respectively. Moreover, Kubota *et al* (1996) reported that hydrolysis γ-PGA in alkaline solution at 90°C were increased fragmented γ-PGA and was easily hydrolyzed amide groups of polymers.

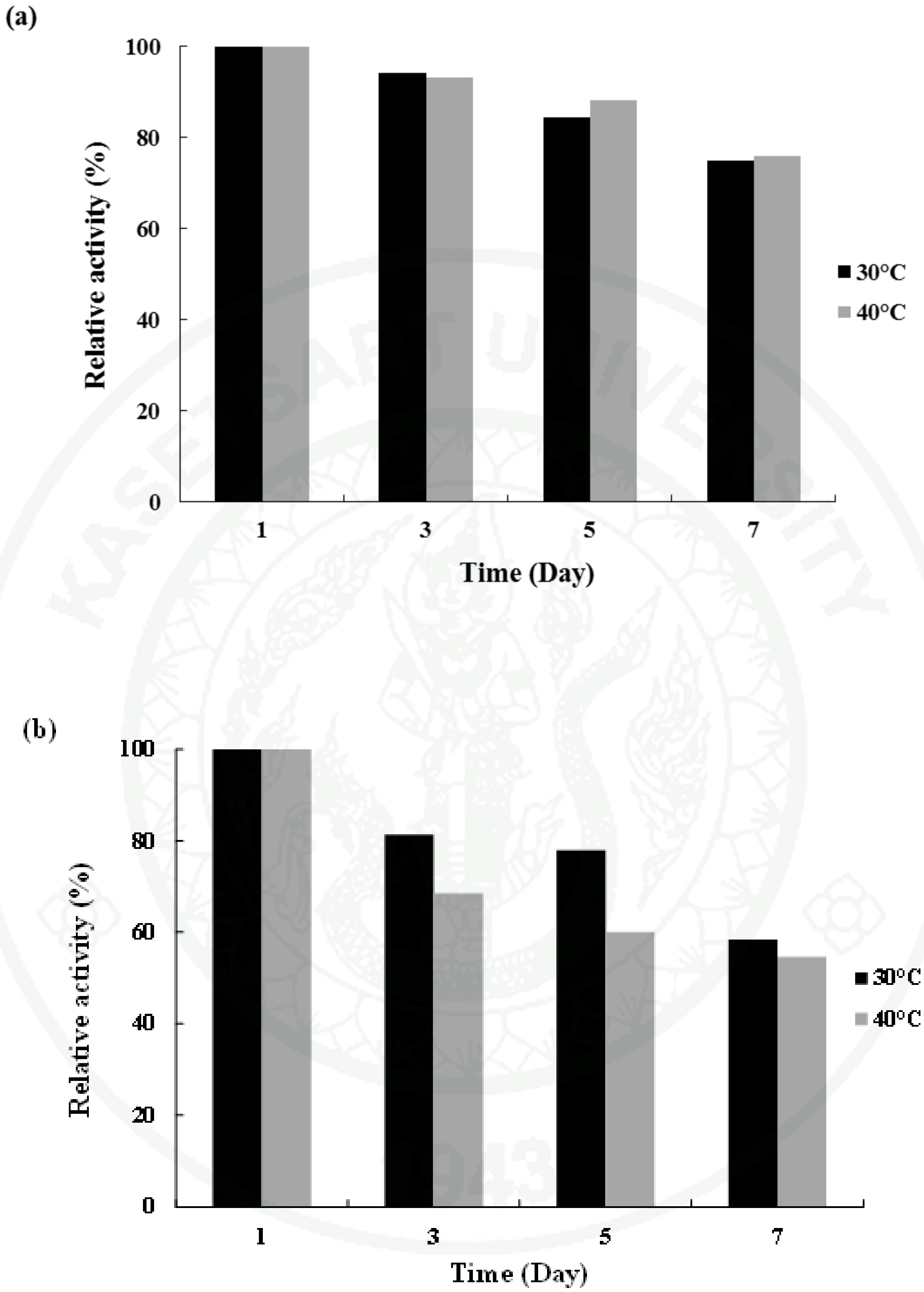
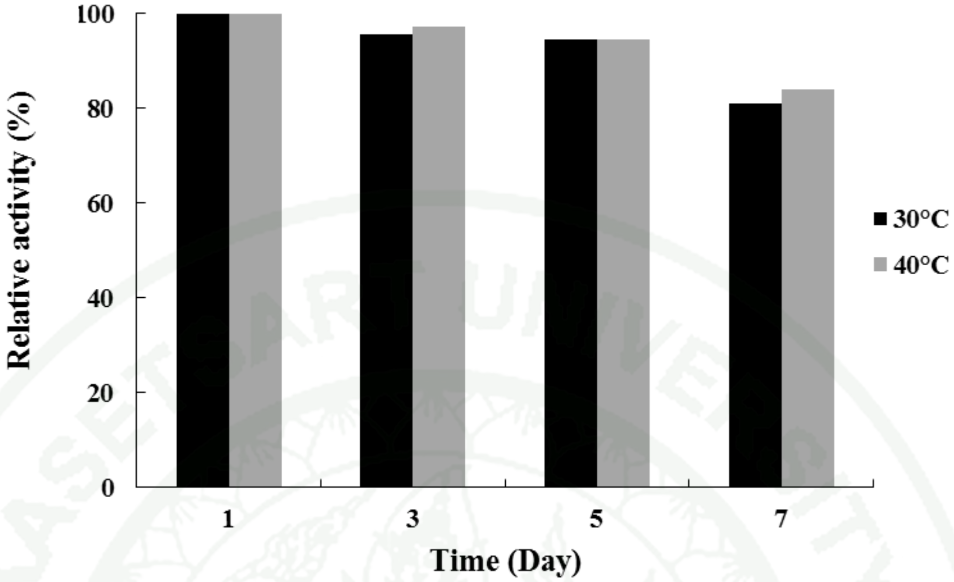


Figure 12 Effect of temperature on stability of γ-PGA from *B. subtilis* NT147 (a) lead biosorption; (b) cadmium biosorption

(a)



(b)

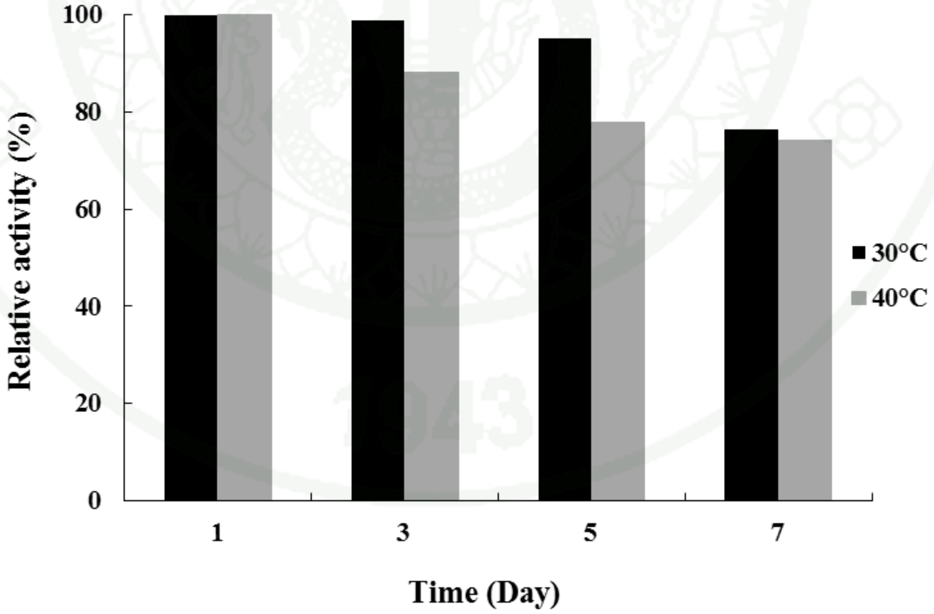


Figure 13 Effect of temperature on stability of γ-PGA from *B. subtilis* NBRC16449 (a) lead biosorption; (b) cadmium biosorption

6. Heavy metal biosorption by biomass cell

6.1 Effect of pH on biomass cell adsorption

Similarly, previous studied have shown that pH is significant factor influencing on heavy metal biosorption. The effect of pH on Pb(II) adsorption was studied and the result were shown in Fig. 14. The percentage removal of Pb(II) by biomass cell of *Bacillus* sp. CR002 increased as the pH of solution increased and approached a maximum Pb(II) uptake at pH 5. Furthermore, there is also no significant variation in the adsorption capacity between pH 4 to 7. However, the experiments were not conducted above pH 7 to avoid possible precipitation lead hydroxide occurs.

The biosorption capacities of cadmium on various pH values are shown in Fig. 15. The experiments were investigated on efficiency removal of Cd(II) by biomass cell of *B. subtilis* MS104. The percentage removal of cadmium increased with increasing pH of solution and was able adsorb up to 72% at pH 6. The results were not different variation in the percentage removal between pH 4 to 7 as shown in Fig. 16. The pH dependence is due to the deprotonation of cell wall functional groups that occurs with increasing pH. However, it has been reported that the precipitation of cadmium at pH above 7 (Namasivayam *et al*., 1995; Ajmal *et al*., 2003).

The effect of pH on biosorption capacity can be explained by the competition of hydronium ion (H_3O^+) and metal ions for binding sites. At low pH values, the ligands such as carboxyl, phosphate and amino groups on cell walls are closely associated with $H_3O^+$and the metal ions is restricted entrance to cell walls by repulsive forces. In contrast at higher pH values, the metal ions may be accumulated inside the cell or cell walls by combined sorption-micro precipitation mechanism.

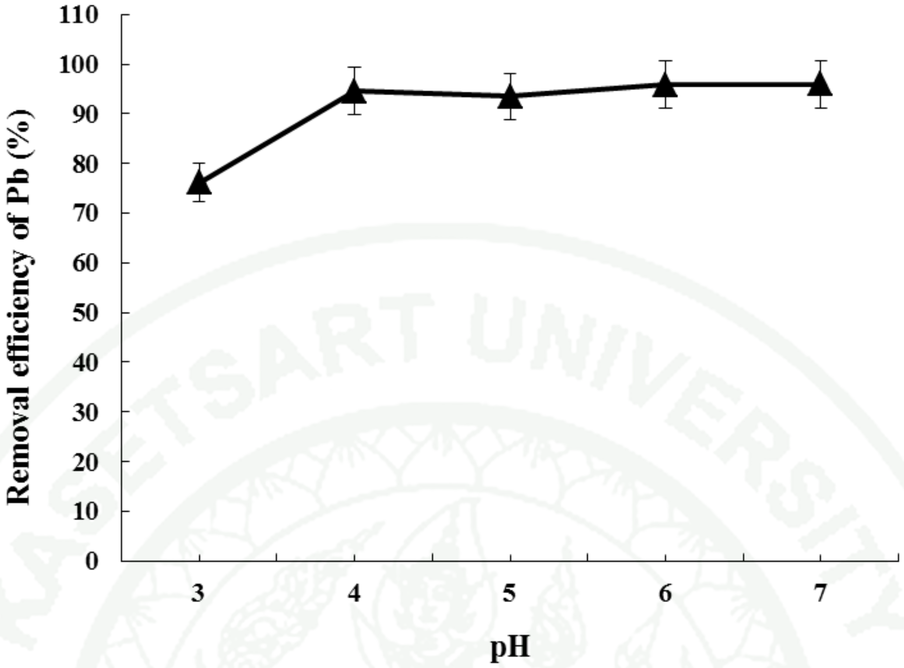


Figure 14 Effect of initial pH on Pb(II) removal efficiency by biomass cell of *Bacillus* sp. CR002 (C_0: 100 mg/L, biomass cell: 0.1g/L, 100 rpm, 30°C

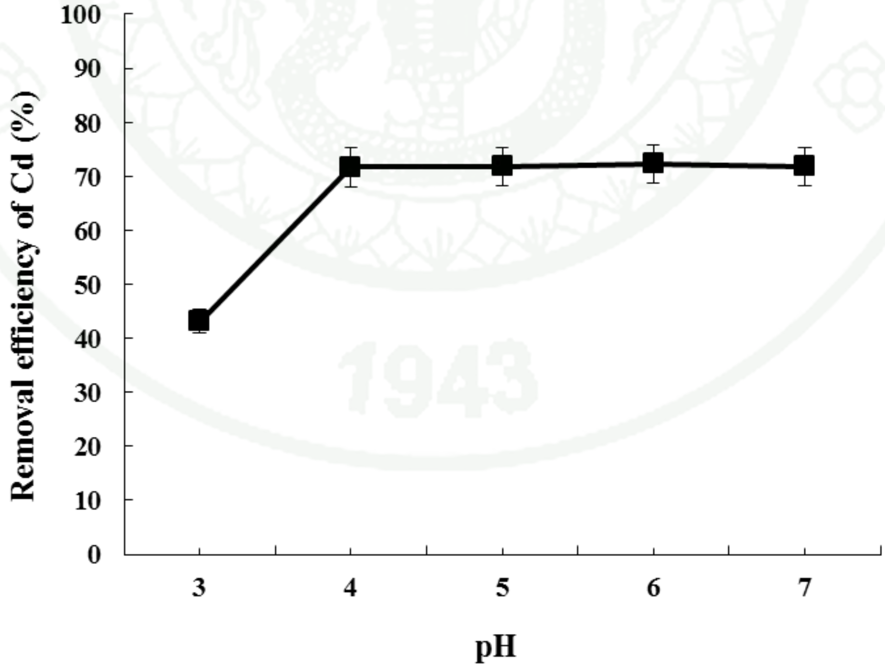


Figure 15 Effect of initial pH on Cd(II) removal efficiency by biomass cell of *B. subtis* MS104 (C_0: 20 mg/L, biomass cell: 0.1g/L, 100 rpm, 30°C)

6.2 Effect of adsorbent mass

The effect of varying the adsorbent mass from 0.25 to 5.0 g/L on the biosorption of Pb(II) and Cd(II) were studied at room temperature (32°C). There are obviously seen that the removal efficiency increases as the biomass increases. Fig. 16 Show that the removal efficiency of Pb(II) 92 % for biomass cell of *Bacillus* sp. CR002 and the maximum specific of Pb was 78.94 mg Pb/g cell at 1g/L cell concentration. The effect of adsorbent dose was studied on Cd(II) adsorption illustrated in Fig. 17. The removal efficiency increases from15.73% to 77.49% as the biomass of *B. subtilis* MS104 increases from 0.25 g/L to 5.0 g/L. The rate of increase gradually decreases with increasing adsorbent mass.

However, as some point that adsorption capacity was decreased or steady with biomass concentration. This could be attributed to interference between binding site at high biomass cells concentrations (De Rome and Gadd,1987) and reduction of metal by biosorbent with increasing of biomass concentration was attributed to an insufficiency of metal ions in solution (Al-Asheh and Duvnjak, 1995).

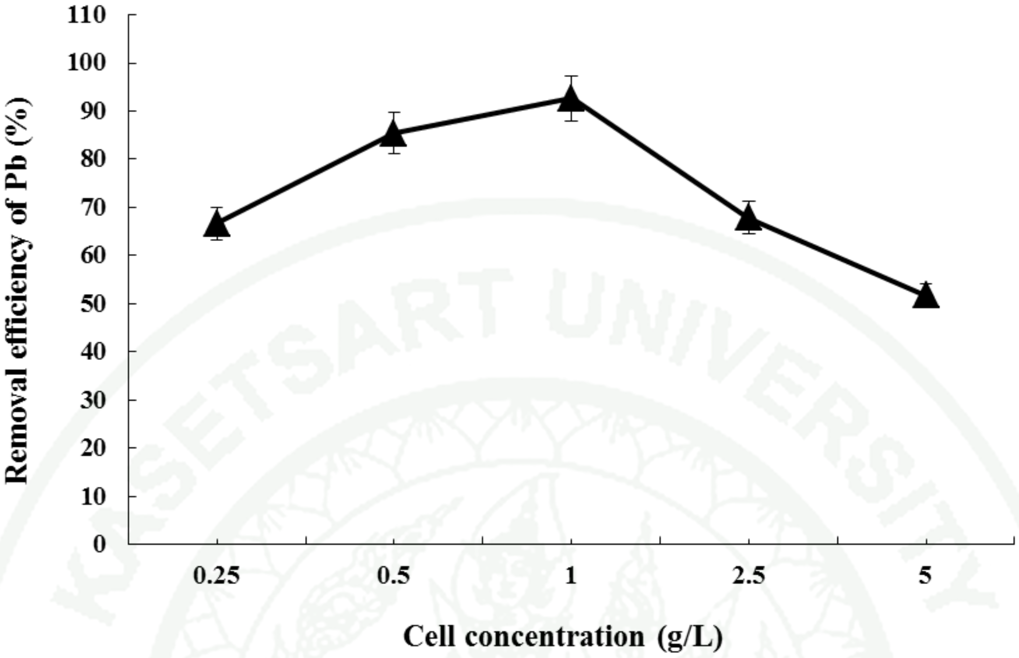


Figure 16 Effect of adsorbent mass on Pb(II) removal efficiency by biomass cell of *Bacillus* sp. CR002 (C_0: 100 mg/L, pH: 5, 100 rpm, 30°C)

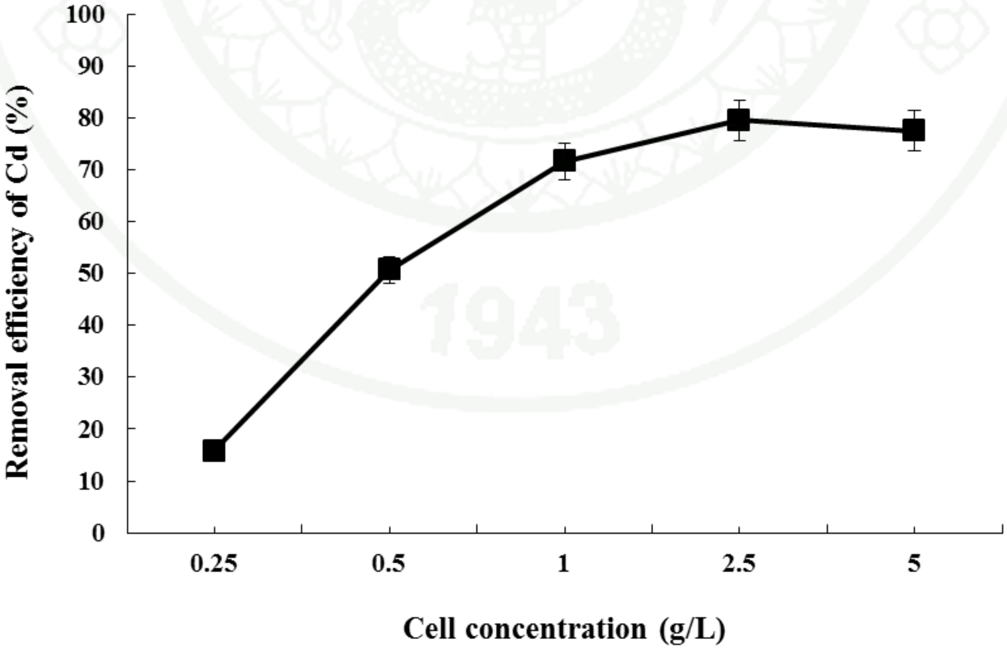


Figure 17 Effect of adsorbent mass on Cd(II) removal efficiency by biomass cell of *B. subtilis* MS104 (C_0: 20 mg/L, pH: 5, 100 rpm, 30°C)

6.3 Effect of temperature on biomass cell adsorption

The effect of temperature on *Bacillus* cells adsorption on Pb(II) and Cd(II) are shown in Fig. 18, 19. The amount of *Bacillus* cells adsorption on Pb(II) and Cd(II) were the least at 25,35 and 45°C, respectively. For Pb(II) adsorption studied, the influence of temperature in the maximum removal of Pb(II) by biomass of *Bacillus* sp. CR002 is insignificant. It seems to have a slight increase as temperature increase. The adsorption rates were probably due to the formation of some new active sites or an increase on the diffusion rate of metal ions from bulk solution to the surface of biosorpbent (Al-Ashed and Duvnjak, 1995).

The adsorption of Cd(II) on *B. subtilis* MS104 cells was obtained at 35°C. Therefore, the greater adsorption of *B. subtilis* MS104 cells measured on Cd(II) solution in the range of temperature from 25 to 45°C. The adsorption decreased from 72.18% to 65.15% for initial cadmium concentration of 20 mg/L with an increase of the temperature from 35 to 45°C. According to an investigation of Kumar *et al* (2010) has been investigated as temperature function on cadmium adsorption on rice husk were decreased from 86.87 to 76.25% at temperatures 25 to 55°C. The result showed that removal efficiency decreased with increasing temperature.

However, possible application of biosorption is moderately to be expected in a temperature range about 5 to 40°C. For high temperatures have affected on the structure of biomass were change or the active sites could be destroyed (Vilar *et al*., 2005).

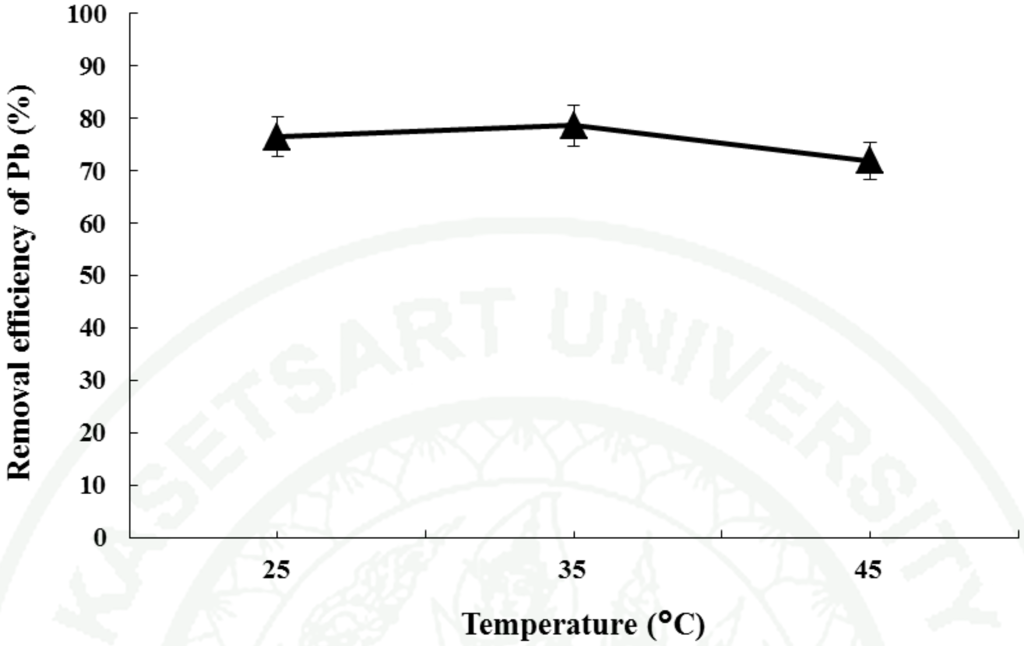


Figure 18 Effect of temperature on Pb(II) removal efficiency by biomass cell of *Bacillus* sp. CR002 (C_0: 100 mg/L, biomass cell: 0.1g/L, 100 rpm)

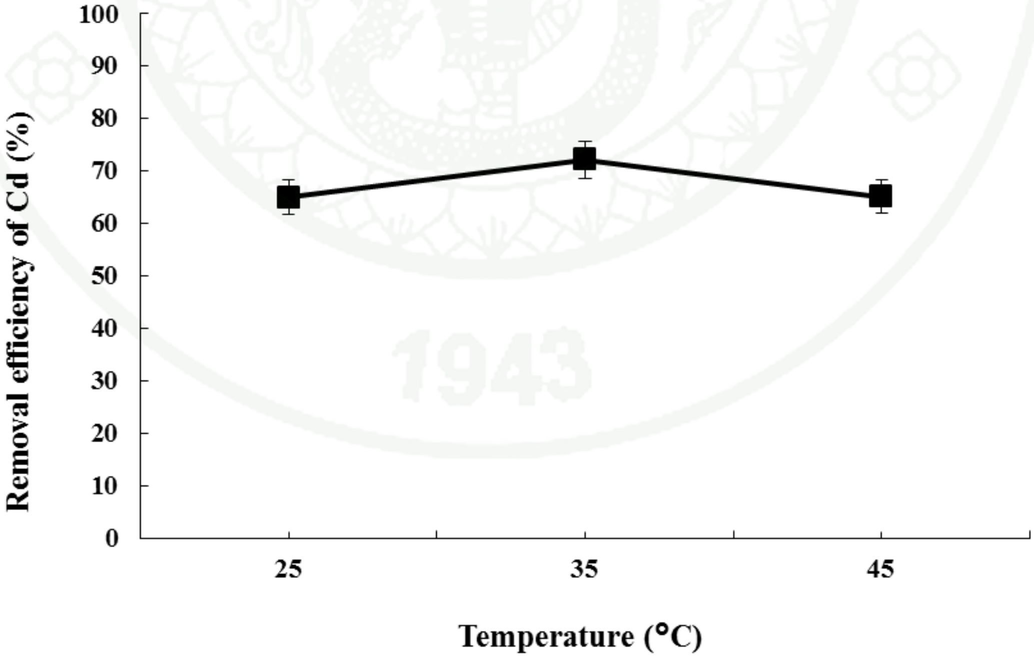


Figure 19 Effect of temperature on Cd(II) removal efficiency by biomass cell of *B. subtilis* MS104 (C_0: 20 mg/L, biomass cell: 0.1g/L, 100 rpm)

6.4 Effect of Initial metal ions concentration on biomass cells biosorption

The increasing an initial metal ions concentration also improves the interaction between metal ions and biosorpbents. Consequently, the initial metal ions concentration enhances diving force to overcome all mass transfer resistances of the metal between aqueous and solid phase (Kumar *et al*., 2009; Kumar *et al*., 2010). The effect of initial concentration of lead at 50 to 700 mg/L and 5 to 100 mg/L for cadmium were evaluated. Table 8 and 9 showed removal efficiency of Pb(II) from the solutions. The efficiency value decreases from 84.35 % to 39.97% for lead and 88.66 % to 44.1% for cadmium, respectively.

In previous study, many factors were reported provide the adsorbate concentration influence that we have been known the important reasons for the adsorption system. The first, the adsorption sites remain unsaturated due to the adsorption reaction. The second cause is the aggregation or agglomeration of biosorpbent particles at higher metal ions concentration (Yasemin and Tez, 2007; Kumar *et al*., 2009; Tangjuank *et al*., 2009). It is clearly seen that as the initial metal ions concentration (table 7, 8) increases that show percentage efficiency removal of metal decreases.

Table 8 Biosorption of different concentrations of lead by *Bacillus* sp. CR002 cells (Biomass cell: 0.1 g/L, 100 rpm, 35°C)

Biosorbent	Initial lead conc. (mg/L)	Residual lead conc. (mg/L)	% Removal of lead	Specific lead uptake(mg/g cell)
Bacillus sp. CR002	46.906	7.338	84.35	39.568
	85.522	10.1791	78.61	75.343
	210.791	49.1	76.70	161.690
	407.992	204.316	49.82	202.877
	634.179	424.776	39.97	209.402

Table 9 Biosorption of different concentrations of cadmium by *B. subtilis* MS104 cells (Biomass cell: 0.1 g/L, 100 rpm, 35°C)

Biosorbent	Initial cadmium conc. (mg/L)	Residual cadmium conc. (mg/L)	% Removal of cadmium	Specific cadmium uptake(mg/g cell)
B. subtilis MS104	4.346	0.492	88.663	3.853
	20.149	0.963	71.572	7.386
	26.326	5.7281	62.349	14.421
	41.707	9.912	59.44	16.414
	96.272	16.915	44.106	24.791

6.5 Adsorption isotherms

The adsorption equilibrium of Pb(II) by *Bacillus* sp. CR002 cells at different concentrations were fitted to the adsorption isotherms of Langmuir. The adsorption isotherm constants of Pb(II) with the corresponding correlation coefficients (R^2) were presented in Table 9. The Langmiur model yielded a good fit to the experimental data (Fig.21) with the highest R^2 of 0.995. Similarly, Vilar *et al* (2005) found that equilibrium on lead biosorption by *Gelidium* and agar extraction from algal waste were fitted to Langmiur isotherm. The biosorption system of Pb(II) on the green alga *Cladophora fascicularis* was reported to also be well modelled by the Langmiur and Freundrich isotherm (Deng *et al*., 2007).

The cadmium sorption isotherm followed the correlation coefficients and isotherm parameters derived from the fitting of experimental -points are given in Table 10. The results show that the experimental data fit both the isotherm equations (Fig. 22). For both metal ions, the Langmuir model yields slightly better fit than the Freundlich model, they were still useful for possibly refining the sorption characteristics. Based on the Freundlich isotherm plot, the adsorption capacity, K_F = 6.2115 mg/g and the adsorption intensity, n = 2.115. From the Langmuir isotherm, the adsorption capacity, Q_0 = 23.47 mg/g and the Langmuir constant, b = 0.4111 L/mg. Therefore, it can be indicated that the polymer in this studied had the great affinity for Pb(II) and Cd(II).

Table 10 Adsorption equilibrium constants for the Pb(II) biosorption by *Bacillus* sp. CR002

Langmiur constants	
Q_0 (mg/L)	250
b (L/mg)	0.0256
R^2	0.995
R_L	0.454
Freundrich constants	
K_F (mg$^{(n\text{-}1)/n}$ L$^{1/n/}$g)	21.2422
n	2.3310
R^2	0.883

Table 11 Adsorption equilibrium constants for the Cd(II) biosorption by *B. subtilis* MS104

Langmiur constants	
Q_0 (mg/L)	23.474
b (L/mg)	0.4111
R^2	0.9853
R_L	0.848
Freundrich constants	
K_F (mg$^{(n\text{-}1)/n}$ L$^{1/n/}$g)	6.2115
N	2.115
R^2	0.9666

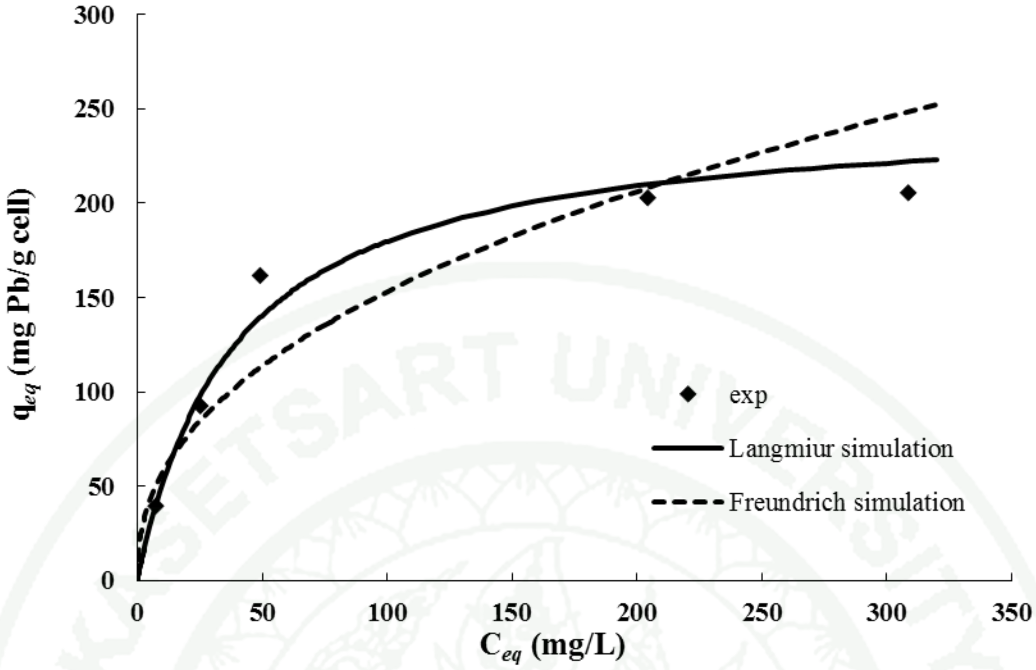


Figure 20 Comparison of adsorption isotherms of Pb(II) by γ-PGA of *Bacillus* sp. CR002

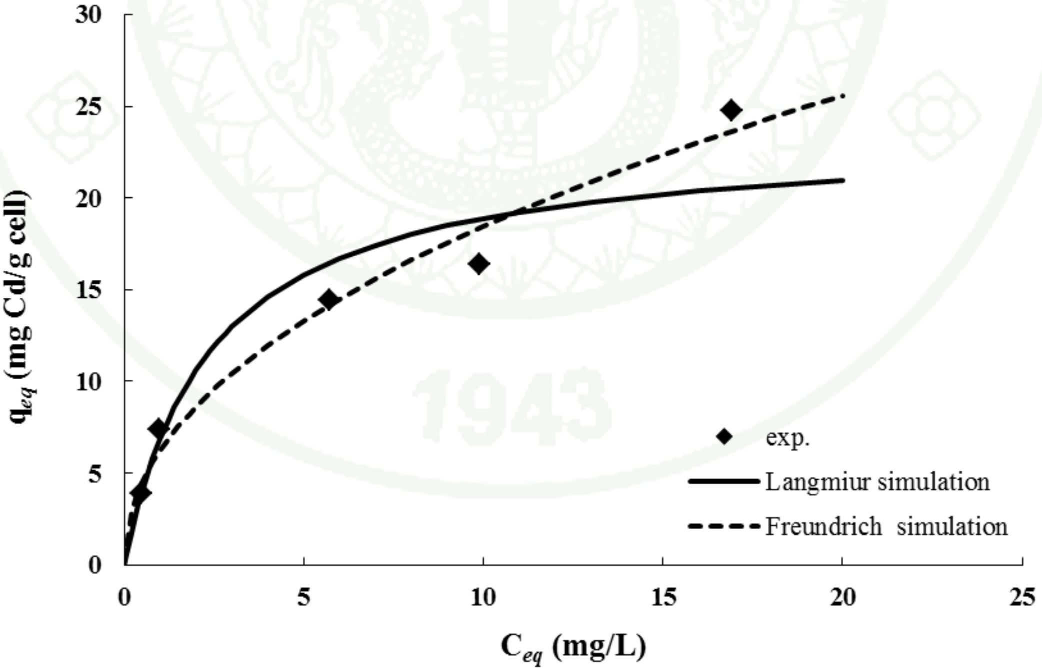


Figure 21 Comparison of adsorption isotherms Cd(II) by *Bacillus* sp. MS104

Table 12 Comparison efficiency of efficiency lead removal between γ-PGA and biomass cell

Biosorbent	Pb(II) concentration(mg/L)	% Pb(II) removal	Specific uptake (mg/g biosorpbent)
γ-PGA of *Bacillus subtilis* NT147	66.094	82.36	54.437
	84.670	79.73	67.514
	235.256	36.17	85.096
	408.653	25.13	102.724
	705.128	18.13	127.724
γ-PGA of *B. subtilis* NBRC16449	70.059	86.52	60.621
	93.821	80.11	81.974
	243.589	35.39	86.217
	411.858	17.76	95.382
	703.205	15.56	109.455
Biomass cell of *Bacillus* sp. CR002	46.906	84.355	39.568
	85.522	78.619	75.343
	210.791	76.706	161.690
	407.992	49.823	202.877
	634.179	39.9	209.402

Table 13 Comparison efficiency of cadmium removal between γ-PGA and biomass cell

Biosorbent	Cd(II) concentration	% Cd(II) removal	Specific uptake (mg/g biosorpbent)
γ-PGA of *Bacillus subtilis* NT147	4.469	29.675	1.326
	7.592	30.381	2.306
	20.676	32.641	6.749
	26.529	27.541	6.500
	37.575	21.573	8.106
γ-PGA of *B. subtilis* NBRC16449	4.202	24.479	1.028
	7.936	21.969	1.743
	20.6391	25.009	5.161
	26.021	23.478	6.109
	49.653	9.906	5.612
Biomass cell *of Bacillus subtilis* MS104	4.346	88.663	3.853
	20.149	71.572	7.386
	26.326	62.349	14.421
	41.707	59.44	16.414

CONCLUSION AND RECOMMENDATION

Conclusion

In this study, we investigated ability of γ-PGA producing bacteria which are able to tolerate and survive in heavy metals stress condition. Biosorption of Pb(II) and Cd(II) by γ-PGA produced from the isolated strains were investigated and factors affecting on biosorption efficiency such as pH, temperature and initial heavy metals concentration and biosorption kinetics were also studied.

1. γ-PGA was isolated from Thai fermented soybean, heavy metals contaminated soil and sludge. The result revealed that 78 isolates obtained from fermented soybean and 20 isolates were obtained from soil are able to produce γ-PGA. The extracellular polymers produced from isolate CR200 and NT147 were characterized. The polymer composes of glutamic acid with D-, L-ratio at 61:39.

2. The isolate CR200 and MS104 were designated as *B. subtilis* based on their morphological, biochemical and genetically achracteristics. *B. subtilis* CR002 showed high level of Pb(II) tolerance and accumulated substantial amount of Pb(II) from the medium. Whereas, *B. subtilis* MS104 was tolerate Cd(II) under the conditions tested.

3. To understand biosorption mechanism γ-PGA produced from isolate NT147 and *B. subtilis* NBRC16449, the biosorption of Pb(II) and Cd(II) ions were studied in ranges pH range from 3.0-7.0. The result exhibited that the optimum pH for biosorption was at pH 5.0. The optimum temperature was at 35°C.

4. For the adsorption equilibrium of γ-PGA of *B. subtilis* CR002 and γ-PGA of *B. subtilis* NBRC16449 the Langmiur isothrem isotherm was found to best describe the biosorption of Pb(II) and Cd(II).

6. For the adsorption equilibrium of the biomass cell, the Langmiur isotherms were found to the best describe the biosorption of Pb(II) and Cd(II).

7. Base on the adsorption equilibrium isotherms, it can be indicated behavior of γ-PGA which demonstrates a heterogeneous nature of Pb(II). However, the Freundlich equation has been limited to the concentration of metals solution.

8. Comparison of quality of binding metals between γ-PGA and biomass cell revealed that biomass cell show a potential adsorbent for remove Pb(II) and Cd(II) from the solutions better than γ-PGA.

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APPENDICES

APPENDIX A

Mediums and preparation methods

Mediums and preparation method

1. Nutrient Agar (NA)

Beef extract	3.0	g
Peptone	5.0	g
Agar	15.0	g

To weigh out, Beef Extract, peptone, agar. First, beef Extract to get a 0.3% solution into the flask, beef extract side down and then peptone and add that to the flask. Add distilled water and swirl to dissolve the peptone and beef extract. Check the pH, it should be 7.0 and add Agar that to the flask.

2. Luria-Bertani medium

Tryptone	10.0	g
Yeast Extract	5.0	g
NaCl	10.0	g

Dissolve components in 1 liter of distilled or deionized water. For LB agar add agar to a final concentration of 1.5%. Heat the mixture to boiling to dissolve agar and sterilize by autoclaving at 15 psi, from 121-124°C for 15 minutes.

3. PGA-productiom medium

Solution I

$(NH_4)_2SO_4$	1.0	g
$Na_2HPO.12H_2O$	0.1	g
KH_2PO_4	0.1	g
Sodium L-glutamic	2.0	g

Solution II Glucose 20 g

Solution III	$MgSO_4$	1.0	g

Solution IV

$CaCl_2$	0.2	g
$MnCl_2$	0.02	g
$FeCl_3$	0.05	g

4 solutions were sterilized by autoclaving at 15 psi, from 121-124°C for 15 minutes. And then the solutions were mixed in to flask 250 ml after that added 0.2 mg/ml of biotin into the mixture solution 250 µl.

4. Minimal salt medium (MSM)

Glucose	1.0 g
K_2HPO_4	7.0 g
KH_2PO_4	2.0 g
Sodium citrate	0.5 g
$MgSO_4 \cdot 7H_2O$	0.1 g
$(NH_4)_2SO_4$	1.0 g
H_2O	500 ml

Mix components in 200 ml of distilled water or deionized water.Use stir bar to mix well. While broth mixes, adjust its pH to 7.5 with dropwise additions of NaOH and HCl. Pour broth into a 500 mL autoclavable flask, at 15 psi, from 121-124°C for 15 minutes.

APPENDIX B

Characterization of bacteria isolated

Appendix Table 1 Isolation and characterization of bacteria from Thua Nao and natto

No.	**Isolate**	**Morphological characteristic**					**Gram stain**	**endospore**	**shape**	**Catalase test**	**PGA producing**
		Form	Elevation	Surface	Edge	Consistency					
1	LP119	Irregular	Flat	Rugose	Curled	membranous	positive	Oval/central	rod shape	positive	+
2	LP120	Irregular	Flat	Rugose	Curled	membranous	Positive	Oval/central	rod shape	positive	+
3	LP124	Irregular	Flat	Rugose	Lobate	membranous	Positive	Oval/central	rod shape	positive	+
4	LP125	Irregular	Flat	Rugose	Undulate	membranous	Positive	Oval/central	rod shape	positive	+
5	LP126	Irregular	Flat	Rough	Undulate	membranous	Positive	Oval/central	rod shape	positive	+
6	LP127	Irregular	Flat	Smooth	Lobate	membranous	Positive	Oval/central	rod shape	positive	+
7	LP128	Irregular	Flat	Smooth	Lobate	membranous	Positive	Oval/central	rod shape	positive	+
8	LP129	Irregular	Flat	Smooth	Curled	membranous	Positive	Oval/central	rod shape	positive	+
9	LP130	Irregular	Flat	Smooth	Lobate	membranous	Positive	Oval/central	rod shape	positive	+
10	LP131	Irregular	Flat	Rugose	Curled	membranous	Positive	Oval/central	rod shape	positive	+
11	LP132	Irregular	Flat	Rugose	Curled	membranous	Positive	Oval/central	rod shape	positive	+
12	LP133	Irregular	Flat	Rugose	Curled	membranous	Positive	Oval/central	rod shape	positive	

Appendix Table 1 Isolation and characterization of bacteria from Thua Nao and natto (Continued)

No.	**Isolate**	**Morphological characteristic**					**Gram stain**	**endospore**	**shape**	**Catalase test**	**PGA producing**
		Form	Elevation	Surface	Edge	Consistency					
13	LP134	Irregular	Flat	Rugose	Curled	membranous	Positive	Oval/central	rod shape	positive	+
14	LP136	Irregular	Flat	Smooth	Lobate	membranous	Positive	Oval/central	rod shape	positive	+
15	LP137	Irregular	Flat	Rugose	Curled	viscid	positive	Oval/central	rod shape	positive	+
16	LP139	Irregular	Flat	Rugose	Curled	viscid	Positive	Oval/central	rod shape	positive	+
17	NT141	Irregular	Flat	Rugose	Undulate	viscid	Positive	Oval/central	rod shape	positive	+
18	NT142	Irregular	Flat	Rugose	Undulate	viscid	Positive	Oval/central	rod shape	positive	+
19	NT143	Irregular	Flat	Rugose	Undulate	viscid	Positive	Oval/central	rod shape	positive	+
20	NT144	Irregular	Flat	Rugose	Undulate	viscid	Positive	Oval/central	rod shape	positive	+
21	NT145	Irregular	Flat	Rugose	Undulate	viscid	Positive	Oval/central	rod shape	positive	+
22	NT146	Irregular	Flat	Rugose	Undulate	viscid	Positive	Oval/central	rod shape	positive	+
23	NT147	Irregular	Flat	Rugose	Undulate	viscid	Positive	Oval/central	rod shape	positive	+
24	NT148	Irregular	Flat	Rugose	Undulate	viscid	Positive	Oval/central	rod shape	positive	+

Appendix Table 1 Isolation and characterization of bacteria from Thua Nao and natto (Continued)

No.	**Isolate**	**Morphological characteristic**					**Gram stain**	**endospore**	**shape**	**Catalase test**	**PGA producing**
		Form	Elevation	Surface	Edge	Consistency					
25	NT149	Irregular	Flat	Rugose	Undulate	viscid	Positive	Oval/central	rod shape	positive	+
26	NT150	Irregular	Flat	Rugose	Undulate	viscid	Positive	Oval/central	rod shape	positive	+
27	CR001	Irregular	Flat	Rugose	Curled	Viscid	Positive	Oval/central	rod shape	positive	+
28	CR002	Irregular	Flat	Rugose	Curled	Viscid	positive	Oval/central	rod shape	positive	+
29	CR003	Irregular	Flat	Rugose	Curled	Viscid	Positive	Oval/central	rod shape	positive	+
30	CR004	Irregular	Flat	Rugose	Lobate	Membranous	Positive	Oval/central	rod shape	positive	+
31	CR005	Irregular	Flat	Rugose	Lobate	Membranous	Positive	Oval/central	rod shape	positive	-
32	CR006	Irregular	Flat	Rugose	Undulate	Membranous	Positive	Oval/central	rod shape	positive	-
33	CR007	Irregular	Flat	Rugose	Undulate	Membranous	Positive	Oval/central	rod shape	positive	-
34	CR008	Irregular	Flat	Rough	Undulate	Membranous	Positive	Oval/central	rod shape	positive	-
35	CR009	Irregular	Flat	Smooth	Lobate	Membranous	Positive	Oval/central	rod shape	positive	-
36	CR010	Irregular	Flat	Smooth	Lobate	Membranous	Positive	Oval/central	rod shape	positive	+

Appendix Table 1 Isolation and characterization of bacteria from Thua Nao and natto (Continued)

No.	**Isolate**	**Morphological characteristic**					**Gram stain**	**endospore**	**shape**	**Catalase test**	**PGA producing**
		Form	Elevation	Surface	Edge	Consistency					
37	CR011	Irregular	Flat	Smooth	Lobate	Membranous	Positive	Oval/central	rod shape	positive	-
39	CR012	Irregular	Flat	Smooth	Lobate	Membranous	Positive	Oval/central	rod shape	positive	+
40	CR013	Irregular	Flat	Smooth	Lobate	Membranous	Positive	Oval/central	rod shape	positive	+
41	CR014	Irregular	Flat	Smooth	Lobate	Membranous	positive	Oval/central	rod shape	positive	+
42	CR015	Irregular	Flat	Smooth	Lobate	Membranous	Positive	Oval/central	rod shape	positive	+
43	CR016	Irregular	Flat	Smooth	Lobate	Membranous	Positive	Oval/central	rod shape	positive	+
44	CR017	Irregular	Flat	Rugose	Lobate	Membranous	Positive	Oval/central	rod shape	positive	+
45	CR018	Irregular	Flat	Rugose	Lobate	Membranous	Positive	Oval/central	rod shape	positive	+
46	CR019	Irregular	Flat	Rugose	Lobate	Membranous	Positive	Oval/central	rod shape	positive	+
47	CR020	Irregular	Flat	Rugose	Lobate	Membranous	Positive	Oval/central	rod shape	positive	+
48	CR021	Irregular	Flat	Rugose	Lobate	Viscid	Positive	Oval/central	rod shape	positive	+
49	CR022	Irregular	Flat	Rugose	Lobate	Viscid	Positive	Oval/central	rod shape	positive	+

Appendix Table 1 Isolation and characterization of bacteria from Thua Nao and natto (Continued)

No.	**Isolate**	**Morphological characteristic**					**Gram stain**	**endospore**	**shape**	**Catalase test**	**PGA producing**
		Form	Elevation	Surface	Edge	Consistency					
50	CR023	Irregular	Flat	Rugose	Lobate	Membranous	Positive	Oval/central	rod shape	positive	+
51	CR024	Irregular	Flat	Rugose	Lobate	Membranous	Positive	Oval/central	rod shape	positive	+
52	CR025	Irregular	Flat	Rugose	Lobate	Viscid	Positive	Oval/central	rod shape	positive	-
53	CR026	Irregular	Flat	Rugose	Lobate	Membranous	Positive	Oval/central	rod shape	positive	+
54	PY001	Irregular	Flat	Rugose	Lobate	Viscid	positive	Oval/central	rod shape	positive	+
55	PY002	Irregular	Flat	Rugose	Lobate	Viscid	Positive	Oval/central	rod shape	positive	-
56	PY003	Irregular	Flat	Rugose	Lobate	Viscid	Positive	Oval/central	rod shape	positive	+
57	PY004	Irregular	Flat	Rugose	Lobate	Viscid	Positive	Oval/central	rod shape	positive	+
58	PY005	Irregular	Flat	Rugose	Lobate	Viscid	Positive	Oval/central	rod shape	positive	+
59	PY006	Irregular	Flat	Rugose	Lobate	Viscid	Positive	Oval/central	rod shape	positive	+
60	PY007	Irregular	Flat	Smooth	Lobate	Membranous	Positive	Oval/central	rod shape	positive	+
61	PY008	Irregular	Flat	Rugose	Lobate	Viscid	Positive	Oval/central	rod shape	positive	+

Appendix Table 1 Isolation and characterization of bacteria from Thua Nao and natto (Continued)

No.	**Isolate**	**Morphological characteristic**					**Gram stain**	**endospore**	**shape**	**Catalase test**	**PGA producing**
		Form	Elevation	Surface	Edge	Consistency					
62	PY009	Irregular	Flat	Rugose	Lobate	Viscid	Positive	Oval/central	rod shape	positive	+
63	PR001	Irregular	Flat	Rugose	Lobate	Viscid	Positive	Oval/central	rod shape	positive	+
64	PR002	Irregular	Flat	Rugose	Lobate	Viscid	Positive	Oval/central	rod shape	positive	+
65	PR003	Irregular	Flat	Rugose	Lobate	Membranous	Positive	Oval/central	rod shape	positive	-
66	PR004	Irregular	Flat	Rugose	Lobate	Membranous	Positive	Oval/central	rod shape	positive	+
67	PR005	Irregular	Flat	Rugose	Lobate	Membranous	positive	Oval/central	rod shape	positive	+
68	PR006	Irregular	Flat	Rugose	Lobate	Membranous	Positive	Oval/central	rod shape	positive	-

+ = PGA produced

- = PGA not produced

Appendix Table 2 Isolation and characteristic bacteria isolate from soil

No.	**Isolate**	**Morphological characteristic**					**Gram stain**	**endospore**	**shape**	**Catalase test**	**PGA producing**
		Form	Elevation	Surface	Edge	Consistency					
1	MS101	Irregular	Raised	Rugose	Undulate	Viscid	positive	Oval/central	rod shape	positive	-
2	MS102	Irregular	Raised	Smooth	Entire	Membranous	Positive	Oval/central	rod shape	positive	+
3	MS103	Irregular	Raised	Rugose	Lobate	Viscid	Positive	Oval/central	rod shape	positive	-
4	MS104	Irregular	Raised	Rugose	Lobate	Viscid	Positive	Oval/central	rod shape	positive	+
5	MS105	Irregular	Raised	Smooth	Undulate	Membranous	Positive	Oval/sub terminal	rod shape	positive	+
6	MS107	Irregular	Raised	Smooth	Undulate	Membranous	Positive	Oval/sub terminal	rod shape	positive	+
7	MS201	Irregular	Raised	Rugose	Lobate	Membranous	Positive	Oval/central	rod shape	positive	-
8	MS202	Irregular	Raised	Rugose	Undulate	Viscid	Positive	Oval/central	rod shape	positive	+
9	MS203	Irregular	Raised	Rugose	Undulate	Membranous	Positive	Oval/central	rod shape	positive	-
10	MS301	Irregular	Raised	Rugose	Lobate	Viscid	Positive	Oval/central	rod shape	positive	+
11	MS302	Irregular	Raised	Smooth	Undulate	Membranous	Positive	Oval/central	rod shape	positive	-
12	MS303	Irregular	Raised	Rugose	Lobate	Viscid	Positive	Oval/central	rod shape	positive	+

Appendix Table 2 Isolation and characteristic bacteria isolate from soil (Continued)

No.	**Isolate**	**Morphological characteristic**					**Gram stain**	**endospore**	**shape**	**Catalase test**	**PGA producing**
		Form	Elevation	Surface	Edge	Consistency					
13	MS401	Irregular	Raised	Rugose	Lobate	Membranous	Positive	Oval/central	rod shape	positive	+
14	MS402	Irregular	Raised	Smooth	Undulate	Membranous	positive	Oval/central	rod shape	positive	+
15	MS403	Irregular	Raised	Rugose	Undulate	Viscid	Positive	Oval/central	rod shape	positive	-
16	MS404	Irregular	Raised	Rugose	Lobate	Viscid	Positive	Oval/central	rod shape	positive	+
17	MS405	Irregular	Raised	Smooth	Undulate	Membranous	Positive	Oval/central	rod shape	positive	+
18	MS406	Irregular	Raised	Rugose	Lobate	Viscid	Positive	Oval/sub terminal	rod shape	positive	-
19	MS407	Irregular	Raised	Rugose	Undulate	Membranous	Positive	Oval/central	rod shape	positive	+
20	MS501	Irregular	Raised	Smooth	Undulate	Membranous	Positive	Oval/sub terminal	rod shape	positive	+
21	MS502	Irregular	Raised	Rugose	Lobate	Membranous	Positive	Oval/central	rod shape	positive	+
22	MS503	Irregular	Raised	Rugose	Lobate	Membranous	Positive	Oval/central	rod shape	positive	-
23	MS504	Irregular	Raised	Rugose	Undulate	Viscid	Positive	Oval/central	rod shape	positive	-
24	MS505	Irregular	Raised	Rugose	Lobate	Viscid	Positive	Oval/central	rod shape	positive	+

Appendix Table 2 Isolation and characteristic bacteria isolate from soil (Continued)

No.	**Isolate**	**Morphological characteristic**					**Gram stain**	**endospore**	**shape**	**Catalase test**	**PGA producing**
		Form	Elevation	Surface	Edge	Consistency					
25	MS506	Irregular	Raised	Rugose	Lobate	Viscid	Positive	Oval/central	rod shape	positive	+
26	MS507	Irregular	Raised	Rugose	Lobate	Membranous	Positive	Oval/central	rod shape	positive	+
27	MS508	Irregular	Raised	Rugose	Lobate	Viscid	positive	Oval/central	rod shape	positive	-
28	MS509	Irregular	Raised	Rugose	Lobate	Viscid	Positive	Oval/central	rod shape	positive	+
29	MS510	Irregular	Raised	Rugose	Lobate	Viscid	Positive	Oval/central	rod shape	positive	+
30	MS511	Irregular	Raised	Rugose	Lobate	Membranous	Positive	Oval/central	rod shape	positive	+
31	MS512	Irregular	Raised	Rugose	Undulate	Membranous	Positive	Oval/central	rod shape	positive	-
32	MS513	Irregular	Raised	Rugose	Undulate	Viscid	Positive	Oval/central	rod shape	positive	+
33	MS514	Irregular	Raised	Rugose	Lobate	Viscid	Positive	Oval/central	rod shape	positive	-

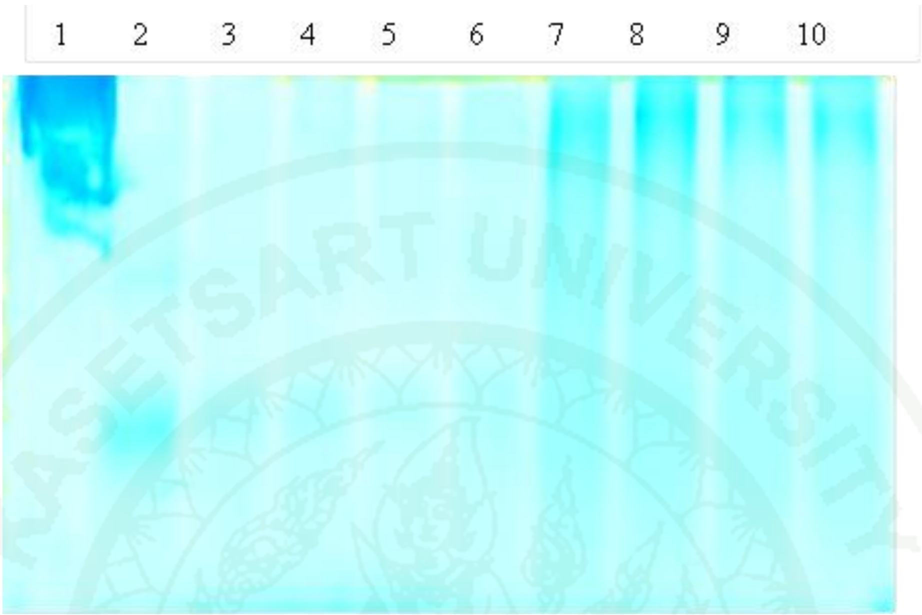
+ = PGA produced

- = PGA not produced

APPENDIX C

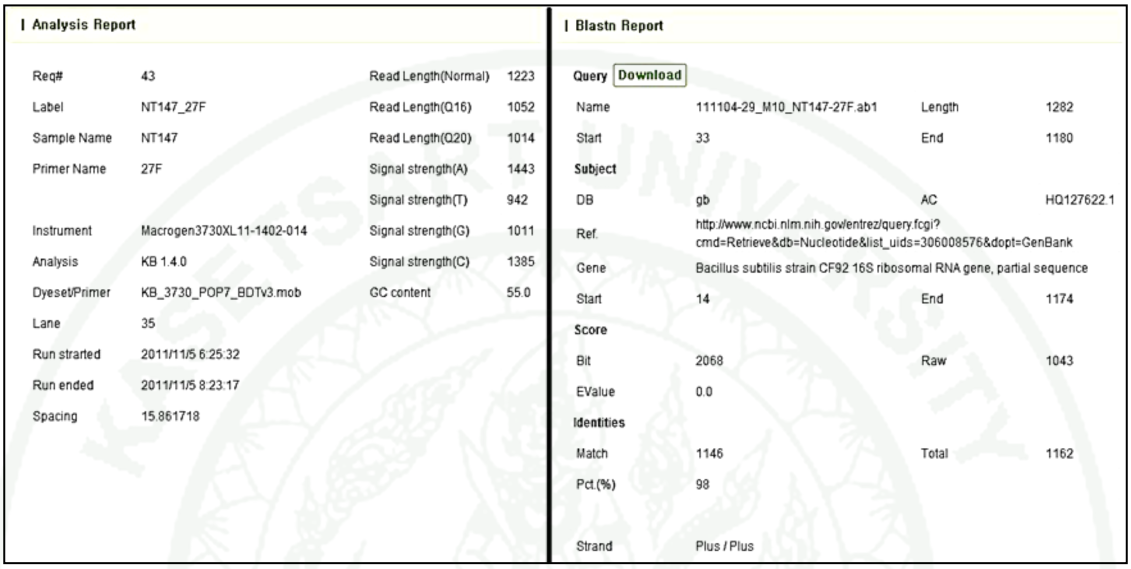
Pictures

Appendix Figure 1 SDS-PAGE analysis of PGA production characteristic of the isolated strians from Thua-nao. Standard PGA 5µg/ml; (1), NT141; (2), NT142; (3), NT144; (4), NT145; (5), NT146; (6), NT147; (7), NT148; (8), NT149; (9) and NT150; (10



Appendix Figure 2 SDS-PAGE analysis of PGA production characteristic of the isolated strains from soil. Standard PGA 5μg/ml; (1), MS101; (2), MS103; (3), MS201; (4), MS203; (5), MS303; (6), MS403; (7), MS404; (8), MS506; (9) and MS511; (10)

Appendix Figure 3 Identification of *B. subtilis* NT147 and *B. subtilis* MS 104 based on 16s RNA

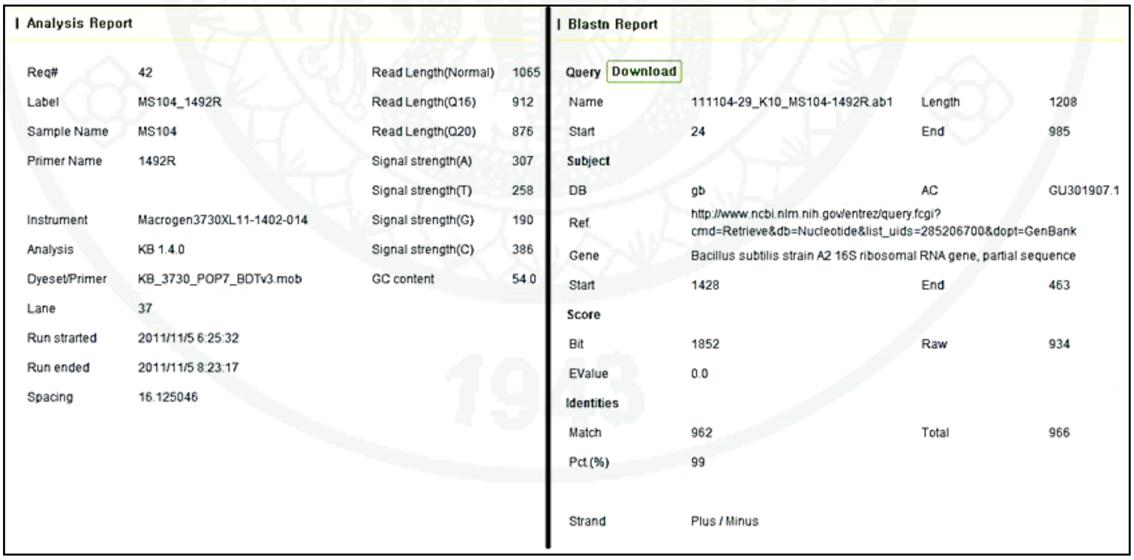


Analysis Report

Req#	43	Read Length(Normal)	1223
Label	NT147_27F	Read Length(Q16)	1052
Sample Name	NT147	Read Length(Q20)	1014
Primer Name	27F	Signal strength(A)	1443
		Signal strength(T)	942
Instrument	Macrogen3730XL11-1402-014	Signal strength(G)	1011
Analysis	KB 1.4.0	Signal strength(C)	1385
Dyeset/Primer	KB_3730_POP7_BDTv3.mob	GC content	55.0
Lane	35		
Run strarted	2011/11/5 6:25:32		
Run ended	2011/11/5 8:23:17		
Spacing	15.861718		

Blastn Report

Query	Download		
Name	111104-29_M10_NT147-27F.ab1	Length	1282
Start	33	End	1180
Subject			
DB	gb	AC	HQ127622.1
Ref.	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi? cmd=Retrieve&db=Nucleotide&list_uids=306008576&dopt=GenBank		
Gene	Bacillus subtilis strain CF92 16S ribosomal RNA gene, partial sequence		
Start	14	End	1174
Score			
Bit	2068	Raw	1043
EValue	0.0		
Identities			
Match	1146	Total	1162
Pct.(%)	98		
Strand	Plus / Plus		



Analysis Report

Req#	42	Read Length(Normal)	1065
Label	MS104_1492R	Read Length(Q16)	912
Sample Name	MS104	Read Length(Q20)	876
Primer Name	1492R	Signal strength(A)	307
		Signal strength(T)	258
Instrument	Macrogen3730XL11-1402-014	Signal strength(G)	190
Analysis	KB 1.4.0	Signal strength(C)	386
Dyeset/Primer	KB_3730_POP7_BDTv3.mob	GC content	54.0
Lane	37		
Run strarted	2011/11/5 6:25:32		
Run ended	2011/11/5 8:23:17		
Spacing	16.125046		

Blastn Report

Query	Download		
Name	111104-29_K10_MS104-1492R.ab1	Length	1208
Start	24	End	985
Subject			
DB	gb	AC	GU301907.1
Ref.	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi? cmd=Retrieve&db=Nucleotide&list_uids=285206700&dopt=GenBank		
Gene	Bacillus subtilis strain A2 16S ribosomal RNA gene, partial sequence		
Start	1428	End	463
Score			
Bit	1852	Raw	934
EValue	0.0		
Identities			
Match	962	Total	966
Pct.(%)	99		
Strand	Plus / Minus		

CURRICULUM VITAE

Personal Information

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Gender	Female
Date of Birth	August 13, 1983
Place of Birth	Satun, Thailand

Educational Background

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2010	Financial source: The Graduate School Kasetsart University

Conferences/Proceeding Presentations

Ruadeerat Boonsanit, Wanwisa Kanpai, Orawan Chunhachart. 2011. Screening of γ-Polyglutamic acid producing bacteria. The 8th Kasetsart University Kumpeang Saen campus conference.

Ruadeerat Boonsanit, Kritchaya Issakul, Rachapol Pawongrat, Wanpen Laohsripaiboon and Orawan Chunhachart. 2011. Biosorption of Pb(II) by lead tolerance *Bacillus* sp. CR002. The 1st Environment Asia International Conference on Environmental Supporting in Food and Energy Security Crisis and Opportunit