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การนำกลับคืนโลหะเงินจากฟิล์มเอ็กซเรย์ที่ใช้แล้วโดยใช้เอนไซม์ คุมาโมไลซิน-เอเอส

RECOVERY OF SILVER FROM USED X-RAY FILMS BY KUMAMOLISIN-AS


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RECOVERY OF SILVER FROM USED X-RAY FILMS BY KUMAMOLISIN-AS

AUTTAPORN BOONJAROEN

King Mongkut's Institute of Technology Ladkrabang

A THESIS SUBMITTED IN PARTIAL FULFILLMENT

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IN BIOTECHNOLOGY

FACULTY OF SCIENCE

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Thesis Title	Recovery of silver from used X-rays films by Kumamolisin-As
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Degree	Master Degree of Science
Program	Biotechnology
Year	2013
Thesis Advisor	Asst. Prof. Dr. Pana Lohasupthawee
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ABSTRACT

The objective of this study was to develop silver recovery process from used X-ray films by using Kumamolisin-As, a collagenolytic serine carboxyl proteinase from *Alicyclobacillus sendaiensis* strain NTAP-1. Kumamolisin-As (K-As) consisted of two activity bands with molecular masses of 41 and 45 kDa and showed hydrolytic activity against various collagen substrates. The optimal temperature, pH and salt concentration for the K-As activity were at 55°C, pH 4.0 and in the presence of NaCl 4 M, respectively. In addition, K-As was found to be stable over a wide range of NaCl (1.5 to 4.0 M) at pH 3.0 to 5.0 and 37 to 80°C which maintained more than 80 % of its original activity. The result implied that K-As is thermostable whilst exhibits its optimum at acidic pH and is activated by high concentration of NaCl. For recovering silver from used X-ray films, effect of four variables including K-As concentration, NaCl concentration, time for hydrolysis and shaking rate were screened by Plackett-Burman's design (PBD). The initial screening showed significant negative effects of NaCl with high confidence level at 99.99% in which the percentage of contribution in the silver recovery process was high (67%). Response surface methodology using central composite design was applied for further optimization of NaCl concentrations and other paramenters. The optimum condition for silver recovery was found to be at pH 4.0, 55°C, 0.15 U/ml of reaction, no NaCl added in the static

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condition for 5 min. Under the optimized condition, about 97% of silver was recovered based on the weight of X-ray films. The K-As could be reused effectively without any significant loss in activity. Overall, this study clearly suggests that the K-As is a potential enzyme for efficient recovery of silver from used X-ray films.

Keywords: Kumamolisin-As, silver recovery, used X-ray films, Plackett-Burman's design, Response surface methodology

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หัวข้อวิทยานิพนธ์	การนำกลับคืนโลหะเงินจากฟิล์มเอ็กซเรย์ที่ใช้แล้วโดยใช้เอนไซม์คุมาโมไลซิน-เอเอส
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บทคัดย่อ

วัตถุประสงค์ของการศึกษานี้ เพื่อพัฒนากระบวนการนำกลับคืนธาตุโลหะเงินจากฟิล์มเอ็กซเรย์ที่ใช้แล้วโดยใช้เอนไซม์คุมาโมไลซิน-เอเอส (Kumamolisin-As, K-As) ซึ่งเป็นเอนไซม์ชนิด serine carboxyl proteinase ที่แยกได้จากแบคทีเรีย *Alicyclobacillus sendaiensis* strain NTAP-1 เอนไซม์คุมาโมไลซิน-เอเอส แสดงกิจกรรมในการย่อยสารตั้งต้นประเภทคอลลาเจนได้หลายชนิด ประกอบด้วยโปรตีนที่มีกิจกรรมย่อยสลายโปรตีน ๒ แถบ ที่มีน้ำหนักโมเลกุลท่ากับ ๔๑ และ ๔๕ กิโลดาลตัน อุณหภูมิ ค่าความเป็นกรด-ด่าง และความเข้มข้นของเกลือโซเดียมคลอไรด์ที่เหมาะสมในการทำงานของเอนไซม์เท่ากับ ๕๕ องศาเซลเซียส ๔ และ ๔ โมลาร์ ตามลำดับ เอนไซม์คุมาโมไลซิน-เอเอส มีความเสถียรต่อเกลือโซเดียมคลอไรด์ความเข้มข้น ๑.๕ ถึง ๔ โมลาร์ ค่าความเป็นกรดด่าง ๓.๐ ถึง ๕.๐ และอุณหภูมิ ๓๗ ถึง ๘๐ องศาเซลเซียส โดยมีกิจกรรมของเอนไซม์ที่เหลือมากกว่าร้อยละ ๘๐ เมื่อเทียบกับกิจกรรมเริ่มต้น จากผลการทดลองแสดงให้เห็นว่า เอนไซม์คุมาโมไลซิน-เอเอส ถูกเร่งกิจกรรมได้ด้วยเกลือโซเดียมคลอไรด์ มีเสถียรภาพที่อุณหภูมิและสภาวะความเป็นกรดสูง ในการศึกษานี้ได้ทำการศึกษาปัจจัยที่มีผลต่อการนำกลับคืนโลหะเงิน ประกอบด้วย ความเข้มข้นของเอนไซม์คุมาโมไลซิน-เอเอส ความเข้มข้นเกลือโซเดียมคลอไรด์ เวลาที่ใช้ในการย่อย และอัตราการเขย่า โดยใช้การออกแบบด้วยโปรแกรม Plackett-Burman's design (PBD) จากการคัดเลือกเบื้องต้นพบว่า เกลือโซเดียมคลอไรด์ส่งผล

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กระทบเชิงลบที่ระดับความเชื่อมั่นร้อยละ ๙๕ และแสดงการมีส่วนร่วมในกระบวนการนำกลับคืนที่สูงถึงร้อยละ ๖๗ ได้ทำการศึกษาความเข้มข้นโซเดียมคลอไรด์ที่เหมาะสมร่วมกับปัจจัยอื่นๆ โดยวิธีการออกแบบการทดลองแบบพื้นที่ผิวตอบสนอง (Response surface methodology, RSM) ด้วยการออกแบบส่วนประสมกลาง (Central composite design, CCD) พบว่า การใช้เอนไซม์คุมาโมไลซิน-เอเอส ความเข้มข้น ๐.๑๕ ยูนิตต่อมิลลิลิตรของระบบการนำกลับคืน ที่อุณหภูมิ ๕๕ องศาเซลเซียส ร่วมกับค่าความเป็นกรดด่างเท่ากับ ๔ ระยะเวลา ๕ นาที ในสภาวะไม่เขย่าและไม่มีเกลือโซเดียมคลอไรด์ เป็นสภาวะที่เหมาะสมต่อกระบวนการนำกลับคืนธาตุโลหะเงินด้วยเอนไซม์คุมาโมไลซิน-เอเอส ภายใต้สภาวะที่เหมาะสมนี้ สามารถนำกลับคืนโลหะเงินได้ร้อยละ ๙๗ เมื่อเทียบกับน้ำหนักฟิล์มเริ่มต้นน นอกจากนั้นเอนไซม์คุมาโมไลซิน-เอเอสยังสามารถนำกลับมาใช้ใหม่ได้อย่างมีประสิทธิภาพโดยไม่สูญเสียกิจกรรมของเอนไซม์ โดยภาพรวมการศึกษานี้ได้แสดงให้เห็นอย่างชัดเจนว่าเอนไซม์คุมาโมไลซิน-เอเอส เป็นเอนไซม์ที่มีศักยภาพสำหรับการนำกลับ คืนธาตุโลหะเงินจากฟิล์มเอ็กซเรย์ที่ใช้แล้ว

คำสำคัญ: คุมาโมไลซิน-เอเอส การนำกลับคืนโลหะเงิน ฟิล์มเอ็กซเรย์ที่ใช้แล้ว Plackett-Burman's - design, การออกแบบพื้นที่ผิวตอบสนอง

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Auttaporn Boonjaroen

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CHAPTER I

INTRODUCTION

1.1 Statement of significance and problem

Silver is one of the widely used elements in industry for various processings (Alexandrova *et al.,* 2001). The majority of this element is consumed in photography processing, electrical and electronic industries, ornament, jewelry and silverware (Lin *et al.,* 2011; Biplop *et al.,* 2010; Asma *et al.*, 2004). It is also widely used for human life. Because of its bacteriostatic properties, silver compounds are often used in filters and other equipment to purify swimming pool and drinking water, used as disinfectants in wastewater treatment, and in the processing of foods, beverages, drugs/drug processing (Karimi *et al.*, 2011; Xianghua *et al.,* 2011). Even though it is not as expensive as gold or platinum, silver is still only present in limited amount in nature (Lin *et al.,* 2011). Along with the decreasing amount of silver from natural resources, the cost of silver productions has risen rapidly and the price of silver in the market has increased constantly. The efficient methods for recovery of silver from waste were generated by the above industries have been reported that 25% of the world's silver needs are supplied by recycling and that 75% of this is obtained from scraps such as photographic waste, X-ray films and jewelry (Nagliboglu 2001; Lin *et al.,* 2011; Zhouxiang *et al.,* 2008). The amount of silver in the used X-ray film varies between 1.5 and 2.0% (w/w) thereby, being a very good source for silver recovery compared to other types of film (Shankar 2010a; Ishikawa *et al.,* 1993; Fujiwara *et al.*,1991.; Khunprasert *et al.,* 2007).

The main technologies being used to leach silver metal from used X-ray films include burning, mechanical or physical and chemical treatments and enzymatic method. The burning method relies on combustion for the recovery of silver, which is time consuming and expensive, as well as results in environment hazards while the use of some chemicals at high temperature poses a serious industrial safety problems. Moreover, the produced gas is not only a serious air pollution but also carries about 3~25% silver into air. The valuable polymer substrate was also destroyed and cannot be recycled (Zhouxiang *et al.,* 2008).

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The enzymatic methods using several microbial proteases are being explored as efficient, cost effective and environmental friendly alternatives for silver recovery from X-ray films. The enzymatic hydrolysis of the gelatin layers on the X-ray films in few minutes enables not only the recovery of the silver but also the polyester base which can be recycled. Most of the proteases used so far for silver recovery are of bacterial origin with only few reports on fungal alkaline protease such as mutant of *Aspergillus oryzae* U1521 (Smarntarn, 2001); *Basidiobolus* N.C.L.97.1.1 (Ingale *et al*, 2002); *Conidiobolus coronatus* (Shankar *et al*, 2010) and *Aspergillus versicolor* by Choudhary (2013). Commercially used enzymes in silver recovery from films are thermostable alkaline proteases from *Bacillus* sp. B21-2 (Fujiwara *et al.,* 1987a,b); *Bacillus* sp. B18′(Fujiwara *et al.,* 1991); *B coagulans* PB-77 (Gajju *et al.,* 1996); *Bacillus* sp. A187P (Masui *et al.,* 1999); *B. sphaericus* (Singh *et al.,* 1999); *B. subtilis* ATCC 6633 (Nagliboglu *et al.,* 2001) and *B. subtilis* MTCC N0-10110 (Ramakrishna *et al.* 2010), *Vibrio* sp. (V26) (Manjusha, 2011) A continuous process for recovery of silver was also reported by Fujiwara *et al.* (1989). The basis of kinetic studies and mechanism of enzymatic hydrolysis of the gelatin layers on X-ray films and the resulting release of silver particles has been studied (Ishikawa *et al.,* 1993).

Kumamolisin-As (K-As), a collagenolytic serine-carboxyl proteinase from *Alicyclobacillus sendaiensis* strain NTAP-1 was investigated for enzymatic hydrolysis of gelatin from waste X-ray films. This procedure relies on the basis of enzyme characteristics, with specificity toward macromolecular substrates such as collagen as well as that gelatin, apart from collagen. It has been shown that the K-As was relaxation of collagen under reaction conditions of low pH and high temperature (60°C, pH 4.0) (Tsuruoka *et al.,* 2003). This enzyme showed considerably higher activity in the presence of NaCl than in its absence. Additionally, sodium chloride component in the solution medium leads to the formation of precipitable silver chloride from the slurry solution. Therefore, silver can be recovered from the hydrolysis solution by replacing the silver with sodium chloride and is often recovered from silver chloride precipitation.

The optimization using statistical method has various advantages of being rapid and reliable in short listing of parameters at varying concentrations, leading to significant reduction in the total number of experiments. There are no reports on statistical optimization of silver recovery by enzymatic method. Therefore, the objective of this study was to verify the feasibility of recovery

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silver metal from used X-ray films by K-As through experimental design by statistical method. In designing the experiments and optimizing the results of experiments, Plackett-Burman Design (PBD) (Plackett and Burman, 1944) and Central Composite Design (CCD) were utilized. Recovery of silver by reduction with NaCl from silver-containing solution was investigated. This work would provide potential alternative protease for silver recovery process and preliminary conditions for further development in the large scale.

1.2 Research objectives

This research aims to study factors affecting activities of Kumamolisin-As and to optimize the condition of silver recovery from used X-ray films by Kumamolisin-As through statistical method to a low cost process.

1.3 Research hypothesis

Kumamolisin-As could be used for recovery of silver from used X-ray films under the optimal condition.

1.4 Expected results

1. The waste X-ray films can be suitably treated by Kumamolisin-As to recover silver under the optimal condition.

2. Addition of NaCl can be effectively used for recovery of silver.

3. Knowledge gained from this study could be used as a model for the recovery of other expensive metals by bio-processing and applied in large scale process.

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CHAPTER II

LITERATURE REVIEWS

2.1 Silver

2.1.1 Characteristics

Silver is a relatively rare metallic element occurring naturally in the pure form and in ores. Silver is a white, soft, very ductile and metaleable with the chemical symbol Ag. Silver has a low crustal abundance and occurs primarily in its principal form of silver sulfide (argentite, Ag_2S), the most abundant, naturally occurring form of silver in the environment. It can exist in several oxidation states, with elemental silver and monovalent silver ion as the most common (ATSDR, 1990). Silver has a molecular weight of 107.87 $g \cdot mol^{-1}$, a density of 10.49$g \cdot cm^{-3}$ at 20°C, and a melting point of 961.93°C (Weast *et al.*, 1988). The other properties of silver metal are shown in Table 2.1. Some of the most common silver compounds used in industry include nitrate, chloride, bromide, acetate, oxide, sulfate, and cyanide (Stokinger, 1981).

Silver has been used for its antimicrobial properties since ancient times. Alexander the Great refused to drink water which had not been stored in silver vessels, and Paracelsus claimed the beneficial properties of silver towards health (Silver *et al.,* 2006). When penicillin was discovered and the era of the antibiotics began, the use of silver for its antimicrobial properties decreased (Klasen, 2000a). Since biocide-resistant strains emerged, the interest to use silver as an antimicrobial agent has risen again (Klasen, 2000b). However, antimicrobial agents based on ionic silver (e.g., silver nitrate) have one major drawback; they are easily inactivated due to an ease to form complex and precipitate, thus having a limited use (Atiyeh *et al.*, 2007). While many medical antimicrobial uses of silver have been supplanted by antibiotics, further research into clinical potential continues.

The main application of silver and silver compounds is to make ornaments, jewelry, high-value table ware, electrical conductors, dental alloys, solder and brazing alloys, paints, mirror production, utensils and currency coins (Asma *et al.*, 2004; Biplop *et al.*, 2010). Nowadays, silver metal is also used in electrical contacts and conductors, in mirrors and in

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catalysis of chemical reactions. Silver compounds are used in photographic materials for film and dilute silver nitrate solutions. Silver is also used for cloud seeding, as an antibacterial agent, and has been used for water purification. Silver may be discharged into surface water by various industries and accumulated in soils from the fallout emissions from coal-fired power plants. The increasing cost of the metal, however, is spurring development of recovery practices (Nordberg and Gerhardsson, 1988; U.S. EPA, 1985).

Table 2.1 Properties of silver

General Properties	
Name, Symbol, Number	silver, Ag, 47
Element category	transition metal
Standard atomic weight	107.8682
Electron configuration	[Kr] 4d 105s1
Electron per shell	2, 8, 18, 8, 1
Physical properties	
Phase	solid
Density (near r.t.)	10.49 $g \cdot cm^{-3}$
Liquid density at m.p.	9.320 $g \cdot cm^{-3}$
Melting point	1234.93 K, 961.78°C, 1763°F
Boiling point	2435 K, 2162°C, 3924°F
Heat of fusion	11.28 $kJ \cdot mol^{-1}$
Heat of vaporization	250.58 $kJ \cdot mol^{-1}$
Molar heat capacity	25.350 $J \cdot mol^{-1} \cdot K^{-1}$
Atomic properties	
Oxidation states	1, 2,3 (amphoteric oxide)
Electronegativity	1.93 (Pauling scale)
Ionization energies	1st: 731.0 $kJ \cdot mol^{-1}$, 2nd: 2070 $kJ \cdot mol^{-1}$, 3rd: 3361 $kJ \cdot mol^{-1}$
Atomic radius	144 pm
Covalent radius	145±5 pm

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Table 2.1 Properties of silver (continued)

Miscellanea	
Crystal structure	face-centered cubic
Magnetic ordering	diamagnetic
Electrical resistivity	(20°C) 15.87 nΩ·m
Thermal conductivity	429 $W \cdot m^{-1} \cdot K^{-1}$
Thermal diffusivity	(300 K) 174 mm^2/s
Thermal expansion	(25°C) 18.9 $\mu m \cdot m^{-1} \cdot K^{-1}$
Young's modulus	83 GPa
Shear modulus	30 GPa
Bulk modulus	100 GPa
Poisson ratio	0.37
Mohs hardness	2.5
Vickers hardness	251 MPa
Brinell hardness	24.5 MPa
CAS registry number	7440-22-4

Source: Applied from wikipedia and Smarntarn, 2001

2.1.2 Compounds

Silver metal is insoluble in water and alkali, but is soluble in nitric acid (HNO_3), hot sulfuric acid (H_2SO_4), and potassium cyanide (KCN). Silver nitrate is used as the starting material for the synthesis of many silver compounds, as an antiseptic and yellow stains for glass. Silver metal is not soluble in sulfuric acid. However, silver reacts readily with sulfur or hydrogen sulfide (H_2S) to produce silver sulfide.

Silver chloride (AgCl) is precipitated from solutions of silver nitrate in the presence of chloride ions, and the others of silver halides used in the manufacture of photographic emulsions. Silver is used in glass electrode for pH testing and potentiometric measurement, and as transparent cement for glass. Silver iodide has been used in attempts to seed clouds to produce rain (Hammond, 2000).

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Silver oxide (Ag_2O) can be produced when silver nitrate solution is treated with base. It has been used in watch batteries as a positive electrode (anode).

Silver carbonate (Ag_2CO_3) is precipitated when silver nitrate is treated with sodium carbonate (Na_2CO_3) (Bjelkhagen, 1995).

Silver fulminate (AgONC), a powerful, touch-sensitive explosive used in percussion caps, is made from reaction of silver metal with nitric acid in the presence of ethanol (C_2H_5OH). Another dangerous explosive silver compound is silver azide (AgN_3), formed by reaction of silver nitrate with sodium azide (NaN_3) (Meyer *et al.*, 2007).

2.2 X-ray films

2.2.1 General of X-ray films

X-ray films must be characteristically different from film used in photography since X-ray exposure is different from light exposure. The application of the film has been mainly used for medical purposes. Because of its importance to medical, X-ray films is manufactured with consistent uniformity and quality, which facilitates standardization of exposure and processing. It was found that 98.5% of imported films are distributed to hospitals, medical institutes, clinics; and medical services, e.g., chest X-rays, mammograms, CT scans, etc. Annually, X-ray films of ca. 2 billions are commercialized around the world, these utilizers produce wastes at their premise and become the primary waste generators. The stocks of silver did not change from years to years. This was due to the fact that a procurement amount of new X-ray films is carried out on an annual basis and the amount of used films, i.e., retained silver primarily depends upon the space of the used film collection area in each hospital (Khunprasert *et al.*, 2007).

The major recording medium used in radiology is X-ray films although the situation is changing with the introduction of new technologies in recent years. The film can be exposed by the direct action of X-rays, but more commonly the X-ray energy is converted into light by intensifying screens and this light is used to expose the film. The basic structure of the film is outlined in Figure 2.1.

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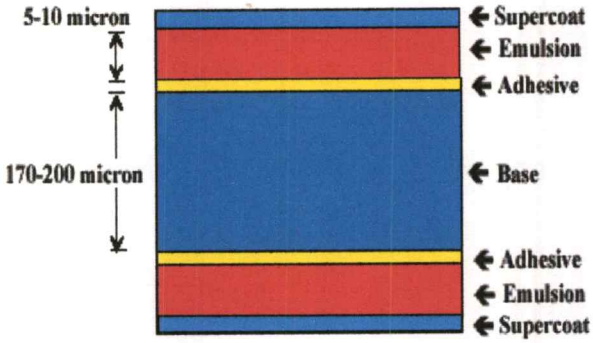

Figure 2.1 Cross-section through a double emulsion film

Available: http://www.e-radiography.net/radtech/f/film.htm

2.2.2 Composition of X-ray films

In general, an X-ray film is a double-emulsion film containing of a transparent base with a adhesive sub-coating, sensitized emulsion, and protective coating on both surfaces.

2.2.2.1 Film base

The film base supports the emulsion and provides the correct degree of stiffness for handling purpose. However, the base must be flexible for ease of processing, essentially be transparent to light and be dimensionally stable over time. Early base materials were glass and cellulose nitrate, but more recently cellulose triacetate and polyester have been used both of which are transparent and blue-tinted.

2.2.2.2 Cellulose acetate

Cellulose acetate is made by dissolving cotton in acetic acid. The cotton cellulose interacts with the acid and form cellulose acetate, which is then dissolved in a volatile solvent containing blue dye. The solution of cellulose acetate is poured onto huge heated drums where it solidifies to thickness of about 0.008 inch. Cellulose acetate does have a tendency to become saturated with liquids during processing, which results in frequent in automatic processors.

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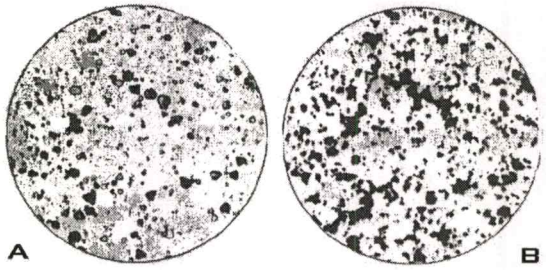

Figure 2.2 Photomicrograph of film emulsion (2500 x)

Available: http://www.tpub.com/content/armymedical/MD0954/MD09540009.html

2.2.2.3 Polyester

The polyester base is made by cracking crude of oil forming the substance parazylene. When this substance is mixed with methyl alcohol and other chemicals, a product of dimethyl-terebthalate (DMT) is formed. This DMT is one-half of the final preparation. Ethylene glycol is the other major ingredient. This is commonly called antifreeze. It is produced from oil and natural gas in a process of oxidizing and hydrolyzing ethylene gas. When DMT and ethylene glycol are mixed in heat for a long period of time, polyester is formed. The polyester is reheated and given at two-way stretch until its thickness is 0.007 inch.

2.2.2.4 Adhesive

A thin layer of adhesive is then applied to the base and this binds the emulsion layer. Covering the emulsion is a thin super-coat that serves to protect the emulsion from mechanical damage.

2.2.2.5 Emulsion

The two most important ingredients of a photographic emulsion are gelatin and silver halide. When examined under a microscope (Figure 2.2), countless tiny crystals of silver bromide embedded in gelatin can be seen. Upon exposure and development, these crystals are changed into irregular clumps and strands of black metallic silver, which all together form the radiographic image. Figure 2.3 shows a cross-section of an exposed and developed emulsion layer. Each emulsion layer is about 0.001 inch thick, but is usually no thicker than 10 mm.

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Since X-rays pass readily through X-ray films and also through intensifying screens an emulsion layer is coated on both sides of the film base. This provides a greater effect upon exposure to X-rays than would be possible with an emulsion coated only on one side. Actually, when intensifying screens are used, the fluorescent light they emit accounts for most of the exposure.

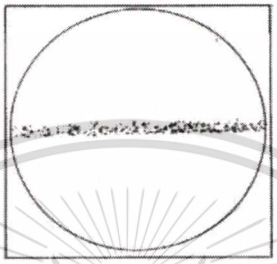

Figure 2.3 Photomicrograph (400 x) of a cross-section of an X-ray films showing an exposed and development emulsion layer (The black particles represent small quantities of black metallic silver)

Available: http://www.tpub.com/content/armymedical/MD0954/MD09540010.html

2.2.2.6 Silver bromide crystal

In the early stages of emulsion manufacturing, a solution of silver nitrate and potassium bromide is produced. This results in the formation of silver bromide crystals and potassium nitrate. The potassium nitrate is then washed out and the result is a silver bromide crystal consisting of bound atoms of silver and bromide. Silver halide is the light sensitive material in the emulsion. In X-ray films, sensitivity is increased by having a mixture of between 1% and 10% silver iodide and 90 to 99% silver bromide. In photographic emulsion the silver halide is suspended in the gelatin as small crystals (called grains). Grain size might average one to 2.3 mm in diameter with up to a billion silver ions per grain and billions of grains per ml of emulsion. In its pure form the silver halide crystal has low photographic sensitivity. The emulsion is sensitised by heating it under controlled conditions with a reducing agent containing sulphur.

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This result in the production of silver sulphide at a site on the surface of the crystal referred to as a sensitivity speck. It is the sensitivity speck that traps electrons to begin formation of the latent image centres. Silver bromide is cream color and absorbs ultraviolet and blue light, but reflects green and red light. Historically, this was fine since the principle emission from calcium tungstate screens is blue light. Films for photography of image intensifier images and films for use with rare earth screens need to have their spectral sensitivity broadened to encompass the longer wavelengths associated with the emissions from these screens. This is accomplished by the addition of suitable dyes. Thus, we have green sensitive orthochromatic film and red sensitive panchromatic film.

2.2.2.7 Gelatin

Photographic gelatin is made from bone and is ideal as a suspension medium in that it prevents clumping of grains. In addition, processing chemicals can penetrate gelatin rapidly without destroying its strength or permanence. Gelatin is a necessary consistent of the X-ray films emulsion. It is an ideal suspension medium for the silver bromide crystals for three primary reasons: It provides an even suspension for the crystals; it has the ability to swell, shrink, and harden; and it increases the sensitivity of the silver bromide crystals.

2.2.2.8 Protective coating

The emulsion is coated with a thin, transparent material to protect it during handling and storage.

2.2.3 Film processing

Film processing is a multi-stage process involving development, fixing, washing and replenishment (Figure 2.4). In development, the exposed grains are preferentially reduced to black metallic silver. In fixing, the remaining unexposed grains are dissolved so that they can be removed from the emulsion by washing. Replenishment ensures that chemical balance is maintained with usage of the processing solutions.

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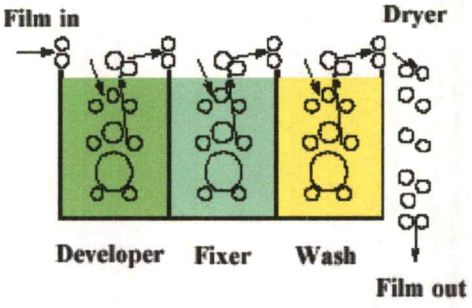

Figure 2.4 Schematic diagram of an automatic film processor, showing the pathway followed by film as it is guided by roller mechanisms through the processing solutions.

Available: http://www.e-radiography.net/radtech/f/film.htm

When the X-ray beam passes through body tissues, variable fractions of the beam will be absorbed, depending on the composition and thickness of the tissues and the quality (kVp & filtration) of the beam. The magnitude of this variation in intensity is the mechanism by which the X-ray beam emanating from the patient produces diagnostic information. The information content of this X-ray image must be transformed into a visible image on the X-ray films with minimal information loss. In general radiography, the X-ray image is first converted to a light image using intensifying screens, which in turn produce a visible pattern of metallic black silver on the X-ray films. Ultimately, the degree of blackening is related to the intensity of the radiation reaching the intensifying screen. The amount of blackness on the film is called the optical density, D, which is defined in Figure 2.5. For example, if 100 light photons are incident on a film and only one is transmitted the film density would be $\log_{10}(100)$ or 2. Useful densities in diagnostic radiology range from about 0.2 to about 2.5. High density means black films.

The curve is a relationship between the logarithm of the radiation exposure and the optical density is plotted, a curve, known as the characteristic curve of the film exposed with an intensifying screen is sigmoidal (Figure 2.6). It is characterized by (i) a toe or region of low gradient at low exposures, (ii) a region of relatively steep increase in density for minimal exposure increases, and (iii) a third relatively flat region called the shoulder at high exposures. The important part of the curve diagnostically is the approximately linear region between the toe and the shoulder where the density is proportional to the logarithm of the exposure.

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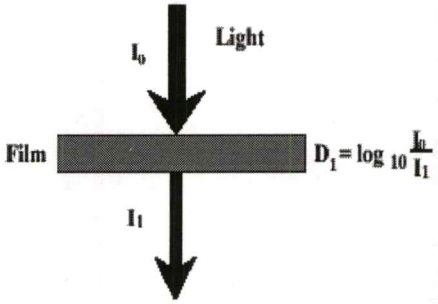

Figure 2.5 The definition of optical density, D

Available: http://www.e-radiography.net/radtech/f/film.htm

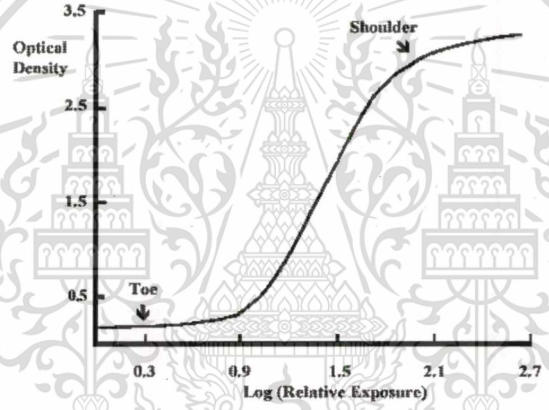

Figure 2.6: The Characteristic curve of X-ray films

Available: http://www.e-radiography.net/radtech/f/film.htm

The information content resulting from the radiograph arises from differences in the film density, which can be defined as radiographic contrast. Radiographic contrast depends on subject contrast and film contrast. The contrast depends on the differential attenuation of the X-ray flux as it passes through the patient and is affected by thickness, density and atomic number of the irradiated parts of the subject, the kVp, the presence of contrast medium and scattered radiation. For example, relatively few X-ray photons pass through bone compared with soft tissue but care must be taken in selecting the correct kVp in order to produce an X-ray image of high information content for the screen-film to record. That is, the kVp influences the magnitude of the

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subject contrast. Film contrast depends on four factors: (i) the characteristic curve of the film, (ii) the film density, (iii) use of intensifying screens or direct exposure and (iv) the film processing.

The slope of the straight line portion of the characteristic curve indicated how much change in film density will occur as exposure changes. The slope or gradient of the curve may be measured and the maximum gradient is called the film gamma, suggesting how well the film will amplify the subject contrast. X-ray films will fog slowly with time, the extent depending markedly on how well it is stored. This fogging, along with the optical density of the film base, will generate a low density in the toe section of the Characteristic Curve. The shoulder region of the curve indicates over exposure.

2.3 Silver recovery techniques from X-ray waste

2.3.1 Production of X-ray wastes

There is currently no film manufacturer in Thailand and X-ray films products are all imported. The application of the film has been mainly used for medical purposes. It was found that 98.5% of imported films are distributed to hospitals, medical institutes, and clinics; these utilizers produce wastes at their premise and become the primary waste generators. The stocks of silver did not change from year to year; this was due to the fact that a procurement amount of new X-ray films is carried out on an annual basis. The amount of used films primarily depends upon the space of the used film collection area in each hospital. Used film and developing solution waste are collected and kept in the hospital area for their annual sale to waste dealers. Wastes in solution form (fixer and developer) are kept for a one-year period; while approximately 87% of hospitals keep used X-rays films for 5-10 years depending on the medical treatment criteria (for personal records) and the size of the collection area (Khunprasert *et al.*, 2008).

After films being used, silver becomes (primary) waste as spent photographic solution and solid sheet material. It was found that, silver, in both metallic and compound forms, remains on the film after being developed and in the spent fixer at 55-65 and 35-45%, respectively. Finally, these wastes are generally sold to waste dealers (WDs) rather than directly to waste processor (WP) for the further recovery of silver. Figure 2.7 show the accessible areas of several in-house waste processors.

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Figure 2.7 Stored of used X-ray films and processing solution waste for silver recovery

Source: Khunprasert *et al.*, 2008

However, a portion of the silver sinks into the environment, through rinse water and used developer solution which are contaminated with silver during the developing process. These solutions are usually drained directly into sewage system with silver concentrations in the range of 12-100 mg/l, which is, according to our best estimation, ca. 0.04 ton of silver (Khunprasert *et al.*, 2007).

Silver, which appears as used component of various products is highly toxic. Investigations on valorization of silver from exposed X-ray films started with onset with photo techniques. Exposed X-ray films have 5-15 g of silver per 1 kg of films, and like that represent significant source of secondary silver. According to the composition of silver content in the film as shown in Table 2.2, an approximate amount of 13 tons of silver enters the country's environmental cycle each year (Khunprasert *et al.*, 2008).

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Table 2.2 Silver contents in materials

Material	Silver content per unit material (gAg/m^2)	Total silver (Ag/kg)
Original X-ray films	4-6[a]	12700[c]
Used X-ray films	3.0-3.5[a]	6980-8320[d]
Solution (fixer+developer)	Around 2000-6000 mg/l[b]	

[a] Material sample is digested with HNO_3 and determined by AAS

[b] Solution sample is directly measured by AAS

[c] Total silver is estimated from 2% Ag on amount of imported X-ray films in 2004

[d] Total silver is estimated from 1.1% to 1.3% Ag on amount imported X-ray films in 2004

Source: Khunprasert *et al.*, 2008

2.3.2 Present techniques of silver recovery from used X-ray films

The common methods for silver recycling from these kind of waste were based on transition of silver into solution, i.e. ionic form, and then by electrolysis producing of silver metal. Processes of production silver from these films could be divided into two groups: mechanical and chemical. Chemical processes could be related to the formation of bearer-gelatin body and dissolving of silver (Marinkovic *et al.*, 2006).

Various studies have been carried out to the technique of silver recycling from used X-ray films or photographic wastes. Figure 2.8 shows the conventional methods being used to leach silver metal from used X-ray films, which are (a) thermal or burning the films directly (Hochberg 1989), (b) biological method by enzymatic hydrolysis of gelatin (see Section 2.4), (c) mechanical or physical (Hochberg, 1989; Buser and Retting, 1988), and (d) chemical treatments to stripping the gelatin-silver layer using different solutions (Moreno, 1986; Messerschmidt, 1988; Laungchonlatan, 1988; Ajiwe and Anyadiegwu, 2000; Syed *et al.*, 2002).

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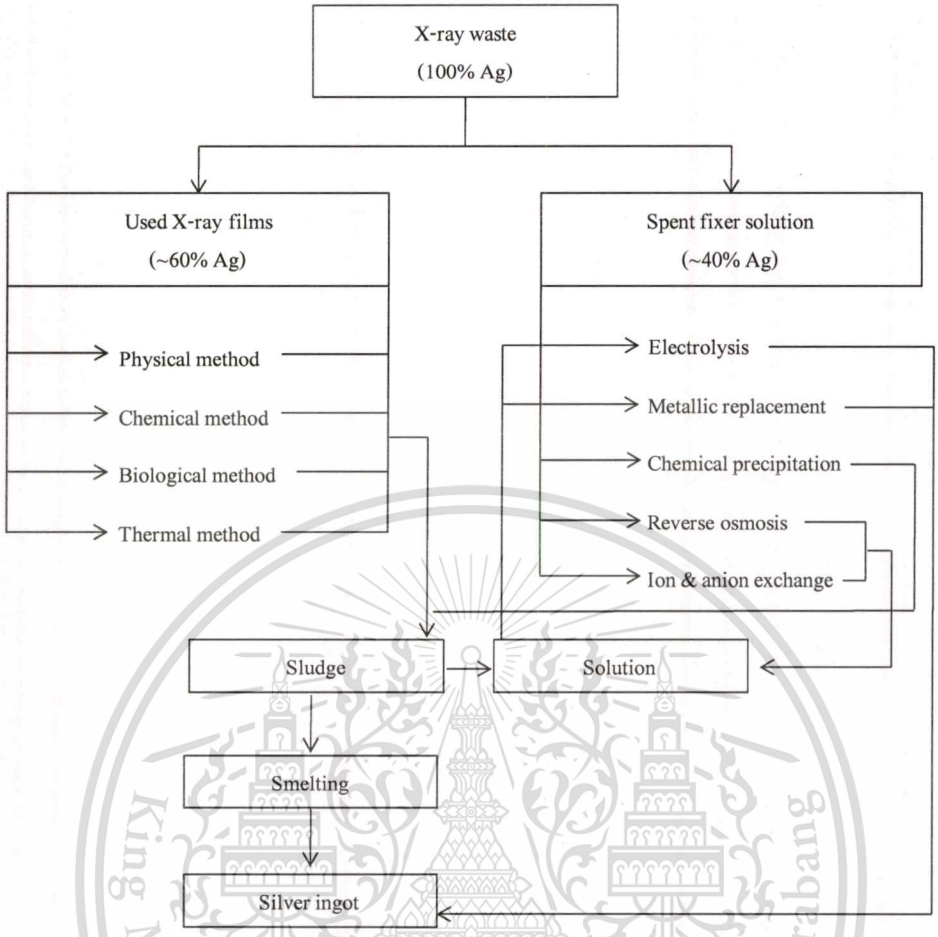

Figure 2.8 Techniques for the recovery of silver recovery from X-ray wastes

Source: Khunprasert *et al.*, 2007

Recovery of silver by thermal or burning the films directly, a conventional method used at present is the most primitive method and generates undesirable foul smell. The method relies on combustion for the recovery of silver and energy which causes environmental pollution. While the rest, not only recover low silver content, but also polyester film on which emulsion of silver and gelatin is coated cannot be recovered. Stripping the gelatin-silver layer by chemical methods using ammonium thiosulphate, sodium thiosulphate, nitric acid or reagents such as sodium cyanide, NaOH, nitric acid or organic compounds cause environmental hazards and are either time consuming or very expensive while the use of NaOH at high temperatures poses a serious industrial safety problem. For this reason, the methods applied to recover silver from X-ray or

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photographic waste should be cost effective and have minimal impact on environment and enzyme based methods can be an alternative option (Shankar *et al.*, 2010; Nagiboglu *et al.*, 2001).

In general, the methods reported for the recovery of silver from films involve two steps; the first is the separation of the silver from the film base, and the second the recovery of the silver by smelting or electrolysis. Electrolysis is effective methods the film base. The silver sludge, obtaining from the latter three methods, is smelted and further refined to form silver ingot.

The first step is commonly leaching, which may be either chemical or microbiological. Table 2.3 shows the techniques for silver recovery from waste photographic films by chemical and mechanical methods. The use of reagents in chemical methods such as sodium cyanide, nitric acid or organic compounds can cause environmental problems. Microorganisms can be used, but this process is slow. On the other hand, all of the existing methods are time-consuming, since they involve two separate steps.

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Table 2.3 Methods for silver recovery from used X-ray films

Process	Solutions	Temperature (°C)	pH	References
Chemicals				
1	$FeCl_3$ (10-26% w/v)	35-40	2	Messerschmidt, 1988
2	$FeCl_3$	NR	NR	Wei and Yue, 2000
3	NaOH (1% w/v)	5-50	alkaline	Parmac *et al.*, 1988
4	NaOH (in EtOH)	boiling solution	alkaline	Chwojnowski and Lada, 1985
5	H_2O_2/NaCN	room temperature	NR	Garcia, 1986
6	EDTA-Fe^{3+} and $(NH_4)_2S_2O_8$)	NR	NR	Yatsugi and Tanaka, 1999
7	$Ce(So_4)_2$	NR	NR	Treshkova S.G. and Prodan E.A., 1988
8	NaClO	room temperature	NR	Nisli *et al.*, 1992
9	HNO_3	room temperature	acidic	Ajiwe and Anyadiegwu, 2000
10	$RCONNH_2$+HNO_3	60-100	acidic	Liu, 1989
11	Urea nitrate (15-20%)	90-95	NR	Guo and W. An, 1987
12	KOH (saturated) + KCl	80	alkaline	Schoenhard, 1980
13	$H_2C_2O_4$ (1% w/v)	97		Syed *et al.*, 2002
14	NaOH or KOH + Na dinaphtyl methane sulfonate (5 g/L)	70-80	alkaline	Sandu *et al.*, 1996
15	Hot water	NR	NR	Lindau, 1970
Mechanical				
16	High shear	140-200 (slurried in water)	NR	Buser and Rettig, 1988
Mechanical and chemical				
17	High shear+NaOH	140-200 (slurried in NaOH solution)	Alkaline	Hochberg, 1989

*Applied from Syed *et al.*, 2002 and Nakiboglu *et al.*, 2003, NR = not report

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2.4. Silver recovery from used X-ray films by enzymatic method

Recovery of silver from waste X-ray films include burning the films directly in a furnace and the silver obtained from the ashes (Ishikawa *et al.*, 1993), oxidation of the metallic silver following electrolysis (Ajiwe and Anyadiewu, 2000) and stripping the gelatin layer containing silver using different chemical solutions (Syed *et al.*, 2002). The conventional method used at present is the most primitive method and generates undesirable foul smell that it is desirable to replace burning by pollution-free methods (Nakiboglu *et al.*, 2001). Moreover, these method causes environmental pollution and polyester film on which emulsion of silver and gelatin is coated cannot be recovered. The stripping of gelatin layer by chemical methods or organic compounds cause environmental hazards and are either time consuming or very expensive while the use of chemical at high temperatures poses a serious industrial safety problem (Sankar *et al.*, 2010). Because of the X-ray structure containing silver and gelatin, it possible to break down the gelatin layer using enzyme and release the silver.

Proteases also have been found potential application in the bio-processing for degrading gelatin coating over the X-ray/photographic waste that releases silver present in the gelatin layer (Joshi and Satyanarayana, 2013). Since the emulsion layer on X-ray films contains silver and gelatin, it is possible to break down the gelatin layer using proteases and release the silver (Nakiboglu *et al.,* 2001). Basically enzymatic processes are more specific and remove gelatin layer from X-ray films without damaging the polyester film base (Gupta *et al.,* 2002; Kumar & Takagi, 1999; Shankar *et al.,* 2010). Hence in recent years, enzymatic methods using microbial proteases are being explored as alternatives to the burning and oxidation methods of silver recovery from photographic/X-ray films (Singh *et al.,* 1999; Ingale *et al.*, 2002; Nakiboglu *et al.,* 2003; Masui *et al.*, 2004). Therefore, an expensive and effluent enzymatic method that does not pollute the environment has been widely suggested to be a sound alternative to the chemical process. A large number of publications report the recoveries of silver from X-ray/photographic waste by enzymatic method are summarized in Table 2.4.

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Table 2.4 Enzymatic methods for silver recovery from X-ray films waste.

Source of protease	Conditions					Silver content	Purity	Reference	Remark
	pH	Temp. (°C)	Enzyme Conc.	Shaking rate (RPM)	Time		(%)		
Bacteria									
Bacillus sp. (B21-2)	10.5	40	100 U/ml	-	10 min	NR	NR	Fujiwara *et al.*, 1987a	isolated from soil
Bacillus sp. (B21-2)	11.5	60	NR	-	NR	NR	NR	Fujiwara *et al.*, 1987b	
Bacillus sp. B18´	10.0	85	1,000 U/ml	-	2 min	NR	NR	Fujiwara *et al.*, 1991	isolated from soil
Bacillus sp. (B21-2) (partial purified)	10.5	40	$2.0 \times 10^{-7}/cm^3$	150	15 min	NR	NR	Ishikawa *et al.*, 1993	carried out in stirred-tank reactor
B. sphaericus	11.0	50	25 U protease/ml	constant shaking	12 min	NR	NR	Singh *et al.*, 1999	isolated from alkaline soil Himalaya
Bacillus sp. B21-2 (mutant Ala187Pro)	10.5	50	5 U/ml	-	60 min	NR	NR	Masui *et al.*,1999	five times repeated with 20 U/ml enzyme
B. subtilis ATCC 6633	8.0	50	22.35 U/ml	-	15 min	NR	99.16±0.16% (mixed with borax)	Nakiboglu *et al.*, 2001	impurities; Co, Fe, Ni, Al, Cu, Cr, Mg, Mn, Sn, Pb, Cd
Bacillus sp. B21-2 (mutant Ala187Pro)	10.5	60	5.6×10^{-7} g/cm^3	150	15 min	NR	NR	Masui *et al.*, 2004	stirred tank reactor
P. aeruginosa	NR	30	NR	100	3 days	NR	NR	Karadzic *et al.*, 2004	X-ray films chip was placed in culture broth
Bacillus subtilis (MTTC N0-10110)	NR	37	5 U/ml (purified)	-	24 h	NR	NR	Ramakrishna *et al.*, 2010	isolated from slaughter house soil
Bacillus RV.B2.90 (partial purified)	NR	37	200 U	-	30 min	NR	NR	Vijayalakshmi *et al.*, 2011	
Vibrio sp. (V26)	9.0	50	1000 U	constant shaking	25 min	NR	NR	Manjusha, 2011	4 runs repeated
rBLAP	12.8	40	5.0 U	-	60 min	NR	NR	Joshi and Satyanarayana, 2013	BLAP gene from *B. lehensis* cloned and expressed in *E.coli*

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Table 2.4 Enzymatic methods for silver recovery from X-ray films waste (continued)

Actinomycete									
S. avermectinus NRRL B-8165 (partial purified)	10	55	NR	-	15 min	NR	NR	Samia *et al.*, 2008	immobilized enzyme
Fungal									
Aspergillus oryzae U1521 (mutant)	9.0	45	10,000 U	250	10 min	94 % (w/w)	NR	Smarntarn, 2001	
Conidiobolus coronatus	10	40	1.35 U/ml	200	6 min	3.87 % (w/w) based on weight of sludge	NR	Shankar *et al.*,2010	4 cycles repeated (with 0.90 U/ml)
Aspergillus versicolor PF/F/107	9.0	50	98.30 U/ml	constant shaking	20 min	0.135 (w/w)	NR	Choudhary, 2013	
Basidioborus N.C.L.97.1.1	10.0	40	50 U/ml	-	6 min	0.1 % (w/w)	99%	Ingale *et al.*, 2002	isolated from plant detritus, Pune, India
Plant									
Bromelain	6.0	55	100 ml crude enzyme	-	35 min	98 %	99.9% (Na_2Co_3, 750 °C, 30 min)	Radha *et al.*, 2010	extract from waste apex pineapple

Note: NR = Not report

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2.5 Kumamolisin-As: A collagenolytic serine-carboxyl proteinase

The serine carboxyl proteases constitute in a new family of S53 enzyme family (now identified in the MEROPS database). Carboxyl proteinases are classified into two groups on the basis of their sensitivity to inhibitors, pepstatin-sensitive and pepstatin-insensitive, which have their optimal pH in acidic region. In 1972 pepstatin-insensitive carboxyl proteinases A, B and C were found in the culture filtrate of *Scytalidium ligicolum*. Carboxyl proteinases have similarity properties to others type of proteinase have been found to be widely distributed among fungi and bacteria (Oyama *et al.,* 2002). Catara *et al.*, 2006 reported a new kumamolisin-like protease from *Alicyclobacillus acidocaldarius*, which active under extreme acidic conditions. More recently, several other enzymes have been classified as member of this family based on sequence similarities. Oda and his colleagues (2012) also reported on serine carboxyl peptidases from *Pseudomonas* sp., sedolisin-B from *Xanthomonas* sp., and Kumamolin-B from *Bacillus coagulans*. This proteinase family also includes eukaryotic homologues, such as the human lysosomal tripeptidyl-peptidase I (CLN2), which has been recently assigned to the family. Mutations in the encoding CLN2 gene are directly associated with a fatal neurodegenerative disease called classical late-infantile neuronal ceroid lipofuscinosis (Bigler, 2004).

Kumamolysin or Kumamolisin is the first of thermostable and pepsin-insensitive carboxyl proteinase from a thermophile of *Bacillus* novo sp. MN-32. In recent years, the crystallographic and modeling studies revealed that the sedolisins (sedolisin, kumamolisin, kumamolisin-As, and CLN2) have an overall fold that is very similar to that of subtilisin. The active sites of these enzymes contain a unique catalytic triad, Ser-Glu-Asp, in place of the canonical Ser-His-Asp triad of the classical serine peptidases (Okubo *et al.*, 2006; Catara *et al.*, 2006).

In 2000, during the course of screening program for thermostable 'acid collagenase' (which has potential applications in biotechnology), Nakayama and co-workers searched for a novel collagenolytic proteinase. They isolated an acidophilic bacterium from soil at Aoba-yama Park, Sendai, Miyagi, Japan, that produces an extracellular thermostable collagenase with high catalytic activity and stability under thermoacidophilic conditions (60°C, pH 4.0), which may reduce the possibility of microbial contamination and should be advantageous during industrial production of the peptides. The peptides obtained from the hydrolysis of collagen by enzyme have potential applications in various of commercial products, such as dietary, preservations, antimicrobial,

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antioxidant, antihypertensive, pharmaceutical supplement and health care materials (Catara *et al.*, 2006) This acidophilic bacterium, strain NTAP-1^{T}, a slightly thermophilic was an aerobic, endospore-forming (Figure 2.9), rod-shaped bacterium that stained gram-negative and was tentatively assigned as a strain of the genus *Bacillus*.

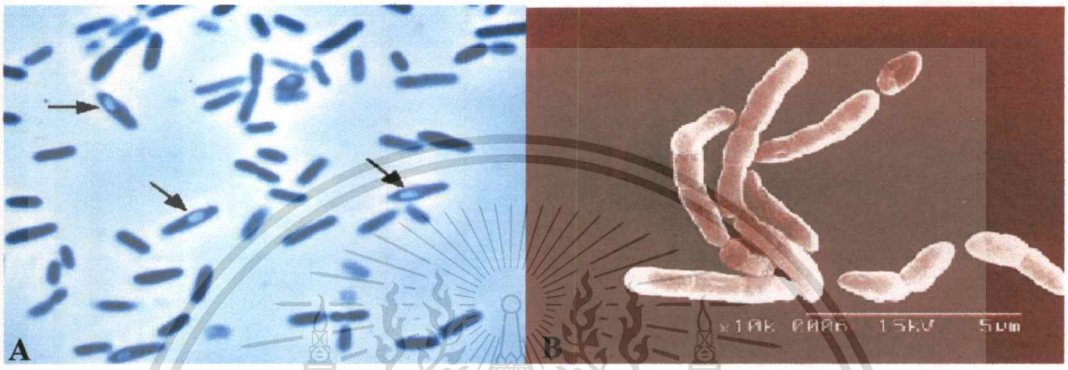

Figure 2.9 Phase-contrast micrograph (A) and scanning electron microscopy (B) of sporulating (↘) and cells of *Alicyclobacillus sendaiensis* strain NTAP-1 sp. nov.. The cells were cultured on BAM liquid medium at 55°C for 3 days.

Source: Tsuruoka *et al.*, 2002, 2003

They have carried out extensive physiological, chemotaxonomic and phylogenetic analyses (Figure 2.10) to indentify and show that strain NTAP-1T represents a novel species of the genus *Alicyclobacillus*, which were have named *Alicyclobacillus sendaiensis* sp. nov.. Then, in 2003, Tsuruoka and co-workers was described the purification to homogeneity, characterization, gene cloning, heterologous expression, and primary-structure analysis of the thermoacidophilic collagenolytic enzyme from *A. sendaiensis* strain NTAP-1 (termed ScpA). The *scpA* gene was expressed under the control of the T7 *lacI* promoter in *E. coli* BL21 (DE3). The recombinant enzyme, rScpA, could be efficiently purified from the crude extracts of transformant cells by a two-step purification procedure: an acid treatment of the extracts at 55°C followed by gel filtration chromatography.

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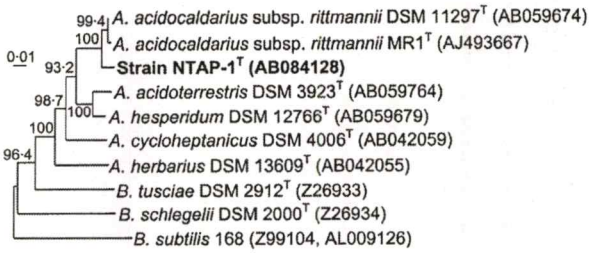

Figure 2.10 Phylogenetic relationships of *Alicyclobacillus* species and some aerobic, rod shape, endospore-forming bacteria, based on 16 rRNA gene sequences. Gen Bank/EMBL/DDBJ accession numbers are shown in parentheses. The branching pattern was generated by the neighbour-joining method. Numbers indicate bootstrap percentages greater than 90%. Bar, 0·01 nucleotide substitution per site.

Source: Tsuruoka *et al.,* 2003

The cloned *scpA* gene encoded a protein of 553 amino acids with a calculated molecular mass of 57,167 Da. (Figure 2.11). Heterologous expression of the *scpA* gene in the *Escherichia coli* BL-21 cells yielded a mature 37-kDa species after a two-step proteolytic cleavage of the precursor protein purified. ScpA is a monomeric, pepstatin-insensitive carboxyl proteinase with a molecular mass of 37-kDa by MALDI-TOF which exhibited the highest reactivity toward collagen (type I, from a bovine Achilles tendon) among the macromolecular substrates examined (Figure 2.12).

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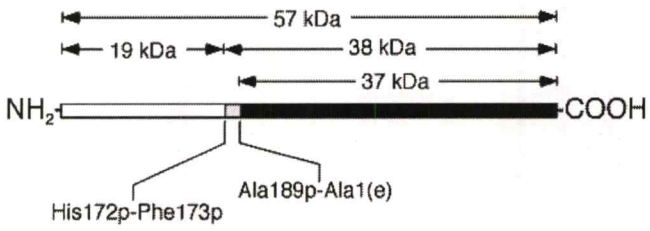

Figure 2.11 Schematic representation of the structure of the precursor of kumamolisin -As, consisting of an N-terminal propeptide (white rectangle), the linker part (gray rectangle), and the mature form of the enzyme (black rectangle). Sizes of the related cleavage products are those estimated from the deduced amino-acid sequences and are shown with double-headed arrows. Cleavage sites are shown below the rectangle.

Source: Okubo *et al.*, 2006

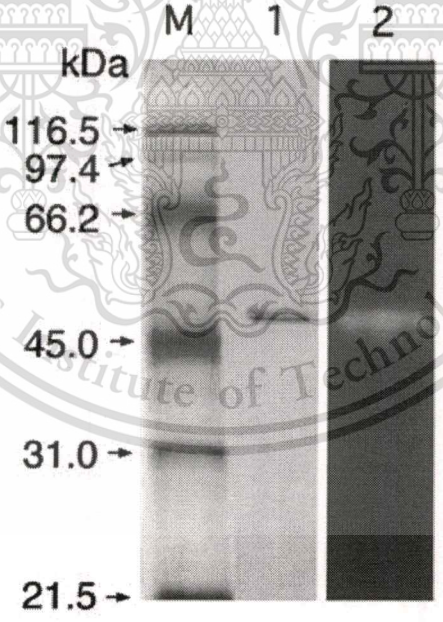

Figure 2.12 Protein pattern and activity staining of purified ScpA from the culture filtrate of *A. sendaiensis* strain NTAP-1. The purified ScpA (lane 1) and marker proteins (lane M) and stained by silver staining. The purified enzyme was also analyzed for gelatinolytic activity by gelatin zymography (lane 2). Molecular sizes of marker proteins are indicated by arrows.

Source: Tsuruoka *et al.*, 2003

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Sequencing of the *scpA* gene revealed that ScpA was a collagenolytic member of the serine-carboxyl proteinase family (the S53 family according to the MEROPS database), which is a recently identified proteinase family on the basis of crystallography results. Unexpectedly, ScpA was highly similar to a member of this family, kumamolysin, whose specificity toward macromolecular substrates has not been defined. Kumamolsin-As (previously called ScpA) an acidic collagenase belongs to the recently characterized family of serine-carboxyl peptidases (sedolisins) that were originally described by Murao, Oda, and co-workers about 20 years ago (Oda *et al.*, 1987; Wlodawer *et al.,* 2003; Tsuruoka *et al.*, 2003). Sedolisins are present in a wide variety of organisms, including archaea, bacteria, molds, slime molds (mixomycetes), amoebas, fishes, and mammals, and they are active at low pH and often high temperature. One interesting question is why nature needs another family of subtilisin-like peptidases. For bacterial sedolisins, one possible explanation is that some organisms might have to utilize such evolved serine-carboxyl peptidases for their survival, presumably under acidic environments and high temperature. The three-dimensional structures are available for three members of the sedolisin family, including sedolisin (also known as pepstatin-insensitive carboxyl proteinase or PSCP), kumamolisin, and kumamolisin-As. The defining features of these enzymes are a unique catalytic triad, Ser-Glu-Asp (Ser278-Glu78-Asp82 for kumamolisin-As) as well as the presence of an aspartic acid residue (Asp164 for kumamolisin-As) (Xu *et al.,* 2007). Specificity analyses using macromolecular substrates including globular and other fibrillar proteins showed that K-As is highly specific for collagen and thus could be considered as a collagenase, although with some unusual properties. Most noticeably, this enzyme exhibits the maximum activity at acidic pH~4.0. This is in striking contrast to all known collagenases, which are either zinc-dependent metallopeptidases or chymotrypsin-like serine proteinases, with an optimum pH for activity at neutral to alkaline regions. The molecular mass, substrate specificity, catalytic properties, and thermostability of the 37-kDa species of rScpA were indistinguishable from those of the native ScpA. The role of its unique catalytic triad could be very interesting issues to be clarified in comparison with the classical types of collagenase.

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2.6 Statistical optimization of process parameters

Optimization of conditions for processing is one of the most critical stages in the development of an efficient and economic bioprocess. Statistical methodologies involved used mathematical models for designing recovery processes and analyzing the response variable value. Design issues are important because they can drastically affect our statistical inference. In the extreme case, a poorly-designed study may not be able to answer the posed scientific hypotheses. Experiments are also increasingly complex and expensive to run in terms of cost and labor. However, there is considerable scope for reducing resources required in research by designing more efficient studies. Careful design considerations even with only minor variation in traditional designs can lead to a more efficient study in terms of more precise estimates or an ability of estimating more effects in the study at the same cost. Factorial designs are popular experimental plans for studying several factors in a scientific investigation. That can be used to detect interactions between two or more factors in an experiment. Several statistical designs are available such as full factorial, fractional factorial of Plackett-Burman design, Taguchi's robust design and response surface methodology (Montogomery, 2001). Although many statistical methods are found in literature, only a few such as PBD, RSM and Taguchi methods have been used in biotechnology processes. Taguchi method is widely used in many engineering design. However, reports of Taguchi methods in biological science are scare because it does not explicitly include model building and optimization (Ross, 1996; Simpson, 1996). A full factorial experiment allows all factorial effects to be estimated independently and is commonly used in practice. However, it is often too costly to perform a full factorial experiment. Instead, a fractional factorial design, which is a subset or fraction of a full factorial design, is often preferred because much fewer runs are required. When this fraction is properly selected, the resulting design has optimal properties. In full factorial designs the number of factors increase exponentially leading to an unmanageable number of experiments. Hence, fractional factorial design like PBD becomes a method of choice for initial screening. Saturated designs are used in the early stages of experimentation to screen out unimportant factors from amongst a large number of possible factors and optimization by RSM.

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2.6.1 Plackett-Burman Design (PBD)

The screening design contains only a sub unit of all possible factor-setting combinations, resulting in fewer required combinations which are ultimately economical and time saving. PBD are popular and well-established widely used statistical method screening for long time ago since, especially in laboratory and pilot plant scale. PBD screens the main factors from a large number of process variables, being a design quite useful in preliminary studies in which the main objective is to select variables that can be fixed or eliminated in further optimization processes. PBD method involves a two level fractional factorial saturated design that uses only n+1 treatment combinations to estimate the main effect of n factors independently (Plackett and Burman, 1946). In particular, PB designs of 11, 19, 23, 27 and 31 factors, all special cases of fractional factorial designs. However, except for obvious cases where some interactions are orthogonal to main effects, analysis of such experiments has been confined to estimating main effects only. This is because of the complex aliasing pattern among main effects and the intricate interactions in these designs. For example, consider the widely used 12-run PBD with 11 factors. There are 55 two-factor interactions and the main effect of each factor X is partially aliased with the 45 two-factor interactions not involving X, thereby making it difficult to disentangle or interpret the significance of interactions. Therefore, PBD are traditionally recommended only for screening purposes under the assumption of additivity of the factor main effects. Each factor in PBD is tested equal number of times and its low and high settings. Because of this equal allocation within each factor, balance exists between each and every pair of factors throughout the design. Equal allocation and balance characterize all two-level designs and make statistically designed experiments complete and efficient from the stand point of resources used and information gained. The screening method of PBD frees the researcher from keeping other factors at constant levels. The large number of components considered for experimentation is varied simultaneously but at different levels. The effects of individual components as well as their significance are conveniently computed mathematically:

$$Y = \beta_0 + \Sigma \beta_I X_{i\,(i = 1,\ldots\ldots\ldots\ldots k)} \tag{2.1}$$

Where: Y is the estimated response, β_0 is the model intercept, $\beta_{I\text{-}k}$ is the linear regression coefficient, $X_{i\text{-}k}$ is the level of the independent variable, which identifies the critical

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physiochemical parameters required for high response levels by screening *n* variables in *n+1* experiments.

The effect of individual parameters on silver recovery was calculated by the following equation:

$$E = (\Sigma M_{+} - \Sigma M)/N \quad (2.2)$$

where E is the effect of parameter under study and M_{+} and M_{-} are responses trials at which the parameter was at its higher and lower levels respectively and N is the total number of trials.

The coefficients obtained from this equation, give information about the impact of each factor individually on the response measured. A number of tools may be used to help access the significance of each factor. These include *p* values, normal plots and pareto charts. The most common means of assessing significance is the *p* value. The *p* value is the probability that the magnitude of a parameters estimate is due to random process variability. A low *p* value indicates a real or significant effect and provides a base line for determining the relative criticality of the variables tested with respect to the response required. Out of these, generally the most showing effective components with positive effect are selected for further study, while those showing large negative effects may be drop for all further experiments. This however is left to the discretion of the investigator depending upon the response studied. Therefore, dual purpose is served with such a systematic approach. Information from screening experiments must first be used fix certain factor settings and possibly revise the design space of the critical factors. All non critical factors i.e. those that will not tested further must be fixed at a constant setting. Typically, the investigator will need to select either middle level of the factor setting range or level at which the factor provides the best response in light of individual effects and interactions observed earlier. After the critical factors have been indentified via screening step, their concentrations are required to be optimized to achieve maximum or mini mum response as per the requirement.

2.6.2 Response Surface Methodology (RSM)

Further optimization can be studies by RSM, is a collection of mathematical and statistical techniques for empirical model building. It is widely used to examine and optimize the operational variables for experiment designing, model developing and factors and conditions optimization. By careful design of experiments, the objective is to optimize a response

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(output variable) which is influenced by several independent variables (input variables). An experiment is a series of tests, called runs, in which changes are made in the input variables in order to identify the reasons for changes in the output response. Originally, RSM was developed to model experimental responses (Box and Draper, 1987), and then migrated into the modelling of numerical experiments. RSM is a powerful mathematical model with a collection of statistical techniques where in, interactions between multiple process variables can be identified with fewer experimental trials. The response is a quantitative continuous variables and the mean response is a smooth but unknown function of levels of k factors. The commonly used response surface designs include Box-Behnken and central composite design (CCD), involving three levels and five levels, respectively, for each factor needed for quadratic terms to be estimable in the second-order model. In general, such a relationship is unknown but can be approximated by a low-degree polynomial model of the form:

$$y = f(x_1, x_2, \ldots x_k) \tag{2.3}$$

where y is the response and $x_1, x_2, \ldots, x_k$ are quantitative levels of the factors interest.

This response is called surface response, which is characterized by a geometric representation obtained when a response variable is plotted as a function of the quantitative factors. The contour plot is series of lines and curves that identify values of the factors for which the response is constant, corresponding to a particular height of the response surface (Figure 2.13). Designs used for fitting response surface are called response surface design.

Usually, one of the RSM problems is that the form of relationship between the response and the independent variables is unknown. Thus, the first step in RSM is to find a suitable approximation for the functional relationship between y and the set of independent variables. Polynomial models of first and second order (linear and quadratic equations with interactions) are normally used to model the response surface. If the response is well modeled by a linear function of the independent variables, then the approximating function is the first-order model.

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \ldots + \beta_k x_k + \epsilon \tag{2.4}$$

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where, y is the response, $\beta_0$is the model intercept and $\beta_{1,..,}$ k is the linear coefficient, and x_i is the level of the independent variable. If there is curvature in the system, then a polynomial of higher degree must be used, such as the second order-model.

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \ldots + \sum\sum_{i<j} \beta_{ij} x_i x_i + \epsilon \qquad (2.5)$$

where y is the predicted response, x_i and x_j the input variables, β_0 a constant, β_i the linear coefficients, β_{ii} the squared coefficients and , β_{ij} the cross-product coefficients.

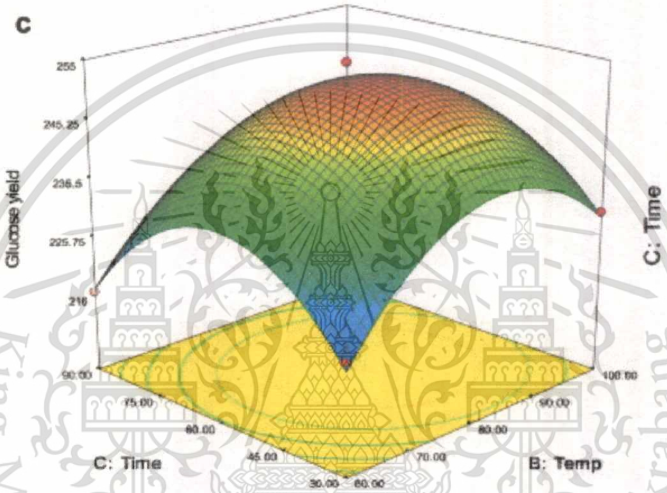

Figure 2.13 Contour plot of a response surface showing the expect yield in function of two variables.

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The RSM is characterized by a sequential procedure and almost all the RSM problems use one or both of these models. Often, when an experience is at a point on the response surface that is remote from the optimum, there is a little curvature in the system and a first-order model will be appropriate. In these cases, the objective is to lead the experimenter rapidly and efficiently along a path of improvement toward the general vicinity of the optimum. Once achieved the optimum region, a more elaborate model (second-order model) may be employed, and an analysis may be performed to locate the optimum profile.

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RSM has important features, such as its sequential approach and its ability to the experimental problem into readily understood geometric terms. It can be applied for different number of variables, generating a mathematical equation to predict improved process conditions. RSM reduces the number of possible combinations to a manageable size, because it uses only a fraction of the total number of factor combinations for experimentation. Thus, the objective of RSM is to determine the optimum operating conditions for the system or to determine the region of the factor space in which operating requirements are satisfied. Therefore, this research carried out researching subject "Silver recovery from used X-rays films by K-As" through statistical methods to obtain high content of silver, contributing to the development of the silver recovery process in general industry in Thailand.

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CHAPTER III

RESEARCH METHODOLOGY

3.1 Bacterial strain

3.1.1 The transformant cells of *Escherichia coli* BL21-(DE3) harboring the plasmid pScpA gene was obtained from Prof. Dr. Oda Kohei of Kyoto Institute of Technology, Japan (Tsuruoka *et al.*, 2003).

3.2 Films

3.2.1. X-ray films (Fuji, Japan)

3.3 Reagents

3.3.1 Antibiotic

3.3.1.1 Amplicillin (Sigma Chemical Co. (St. Louis, MO, USA) (Appendix A)

3.3.2 Culture media

3.3.2.1 Luria-Bertani (LB) medium and Luria-Bertani agar (LBA) (Appendix B)

3.3.3 Chemicals

3.3.3.1 Acrylamide (Bio-rad Laboratories, Inc., Japan)

3.3.3.2 Amido black (Wako, Wako Pure Chemical Industries, Ltd., Japan)

3.3.3.3 Ammonium persulfate (Promega, USA)

3.3.3.4 BME, β-mercaptoethanol (Sigma Chemical Co. (St. Louis, MO, USA)

3.3.3.5 Bovine serum albumin (Sigma Chemical Co. (St. Louis, MO, USA)

3.3.3.6 Bromophenol blue (Sigma Chemical Co. (St. Louis, MO, USA)

3.3.3.7 Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Hercules, CA, USA)

3.3.3.8 Ethanol (Carlo erba, Val de Reuil, France)

3.3.3.9 Gelatin (Lab Chem, Zelienople, PA)

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3.3.3.10 Glacial acetic acid (Merck, Germany)

3.3.3.11 Glycerol (J.T. Baker, USA)

3.3.3.12 Glycine (Sigma Aldrich, Co., St. Louis, MO, USA)

3.3.3.13 Hydrochloric acid (Merck, Germany)

3.3.3.14 Isopropyl-β-D-thiogalactopyranoside, IPTG (Fermentas, USA) (Appendix A)

3.3.3.15 L-leucine (Sigma Aldrich, Co., St. Louis, MO, USA)

3.3.3.16 Methanol (Rci-Labscan, Thailand)

3.3.3.17 N, N, N′, N′-tetramethylethylenediamine, TEMED (Bio-Rad Laboratories, Hercules, CA, USA),

3.3.3.18 Nitric acid (Lab-Scan, Gliwice, Poland)

3.3.3.19 Sodium acetate, CH_3COONa (BDH Laboratories, England)

3.3.3.20 Sodium chloride (Carlo erba, Val de Reuil, France)

3.3.3.21 SDS, Sodium dodecyl sulfate ($NaDodSO_4$) (Bio-Rad Laboratories, Hercules, CA, USA)

3.3.3.22 Sodium hydroxide (Merck, Germany)

3.3.3.23 Sulphuric acid, H_2SO_4 (Merck, Germany)

3.3.3.24. TNBS 2, 4, 6-trinitrobenzenesulfonic acid (Sigma-Aldrich, Co., St. Louis, MO, USA)

3.3.3.25 TritonX-100 (Sigma-Aldrich, Co., St. Louis, MO, USA)

3.3.3.26 Trizma base (Sigma-Aldrich, Co., St. Louis, MO, USA)

3.3.3.27 Tryptone (Difco Laboratories, USA)

3.3.3.28 Wide range molecular weight protein markers (Sigma-Aldrich, Co., St. Louis, MO, USA)

3.3.3.29 Yeast extract (Merck, Germany)

3.3.4 Kits

3.3.4.1 Plus One™ Silver Staining kit, Protein (GE Healthcare, Sweden)

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3.4 Instruments

3.4.1 Autoclave (Hi clave HV-00, Hirayama, Japan)

3.4.2 Balance (AB 204-S, Mettler Toledo, Switzerland)

3.4.3 Balance (PB 3002-S, Mettler Toledo, Switzerland)

3.4.4 Block heater (Bibby Sterilin, LTD, UK)

3.4.5 Boiling bath (Applied Medic, Thailand)

3.4.6 Electrophoresis equipment (AE-6530, ATTO Corporation, Japan)

3.4.7 Floor standing Incubator (Certomat BS-1, Satorius Certomat, Germany)

3.4.8 Glass wares (Pyrex, Germany and Duran, Germany)

3.4.9 Hot plate stirrer (C-MAG HS7, IKA labortecchink, Germany)

3.4.10 Laminar air flow cabinet CLASS II (Nuaire, USA)

3.4.11 Magnetic stirrer (C-MAG MS7, IKA labortecchink, Germany)

3.4.12 pH meter (Seven Easy, Mettler Toledo, Switzerland)

3.4.13 Refrigerated centrifuge (5810R, Eppendorf, Germany)

3.4.14 Scanning electron microscope (JSM-5410LV, JEOL, Tokyo, Japan) with Energy dispersive spectrometry (Link ISIS 300, Oxford instruments, USA)

3.4.15 Ultrasonication (Vibra Cell VCX60, Sonics and Materials Inc., USA)

3.4.16 UV-Vis spectrophotometer (Helios Alpha Thermo Electron Corporation, UK)

3.4.17 Vacuum Centrifuge (Avanti J-E, Beckman-Coulter, Inc., Fullerton, CA, USA)

3.4.18 Vortex mixer (G-560E Vortex Genie 2, USA)

3.4.19 Water bath (Memmert, WNB 7-45, GmbH+Co., Schwabach, Germany)

3.4.20 Water bath (Memmert, WNB 7-45, GmbH+Co., Schwabach, Germany)

3.4.21 X-ray fluorescence spectrometer (PANalytical, PW 2404, Philipps, Netherlands)

3.5 Methods

3.5.1 Microorganism preservation

The stock culture of transformant *E. coli* BL-21(DE3) harboring the plasmid pScpA gene was maintained on Luria-Bertani (LB) broth supplemented with 50 μg/ml of ampicillin and 15% (v/v)

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glycerol stored at -80°C. The lineage was replicated in Luria-Bertani agar (LBA, Appendix B) supplemented with 50 μg/ml of ampicillin and incubated at 37°C for 24 h.

3.5.2 Pre-inoculum and culture conditions

The culture condition was performed according to the method of Tsuruoka *et al.* (2003). A single colony of fresh bacterium was inoculated into 5 ml of LB medium containing 50 μg/ml of ampicillin and incubated at 37°C with shaking at 200 rpm for 18 h. One percent of the pre-inoculum suspension was inoculated into a 1000 ml Erlenmeyer flask containing 300 ml of LB medium supplemented with 50 μg/ml of ampicillin and again incubated at 37°C with shaking at 200 rpm until the A_{600} reached 0.6. Expression of the *scpA* gene was attained by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.8 mM, followed by further cultivation for 3 h. The cells was harvested by centrifugation (5,000 x g at 4°C for 10 min) and then kept the cells pellet at -20°C for further studies.

3.5.3 Preparation of crude K-As

The procedure of Tsuruoka *et. al.* (2003) was used for crude K-As preparation. The cell pellets were suspended in an appropriate volume (5 ml) of a 0.05 M sodium acetate buffer, pH 4.0, and disrupted at 4°C by 5-7 cycles of ultrasonication (at 10 kHz for 20 sec followed by an interval of 20 sec). The cell debris was removed by centrifugation at 18,000 x g at 4°C for 10 min, and the resultant supernatant (pH 4.0) was incubated in water bath at 55°C for 5 h, followed by centrifugation. Almost all of the endogenous *E.coli* proteins after acid treatment were precipitated by centrifugation, and the resultant supernatant contained the 37-kDa species of rScpA with 96% homogeneity was used as crude K-As for further studies.

3.5.4 K-As activity assay

K-As activity was assessed by the method as previously reported by Khantaphant and Benjakul (2008) with slight modification. Briefly, the reaction mixtures contained 880 μl of 0.05 M of Na-acetate buffer, pH 4.0, 100 μl of 2% (w/v) gelatin solution. The reaction mixture without the enzyme was previously incubated at 55°C for 15 min, and the hydrolysis reaction was started by the addition 20 μl of the crude K-As. After incubation at 55°C for 60 min, the homogenates were fully inactivated by placing the mixture in boiling bath for 5 min and cooled down at ambient temperature

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for 15 min. The control blank was prepared in the same manner and reaction was stopped immediately after the crude K-As was added. After that, the appropriate dilution (125 µl) was added with 2.0 ml of 0.2 M phosphate buffer (pH 8.2) and 1.0 ml of 0.01% TNBS solution, mixed thoroughly and placed in a temperature controlled water bath at 50 °C for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulfite. The mixtures were cooled at room temperature for 15 min and the absorbance was read at 420 nm. One unit of enzyme was defined as 1 mM of free α-amino acid in terms of L-leucine was released at time *t* (1 h) under these assay conditions. The specific activity was expressed in units per milligram of protein (Benjakul and Morrissey, 1997).

3.5.5 Characterization of K-As

3.5.5.1 Effect of temperatures and thermostability on K-As activity

The effect of temperatures on K-As activity was estimated at different temperatures (37-80°C) pH 4.0 for 1 h. The K-As activity was determined using K-As activity assay conditions (see section 3.5.4).

The thermostability of K-As was determined by pre-incubated crude K-As in 0.05 M Na-acetate buffer pH 4.0 over the temperature range of 37 -80°C for 1 h. After this period, the enzyme solution was immediately cooled down in an ice bath to stop the effect of temperature. Subsequently, the remaining activity of K-As was measured by using 2% (w/v) gelatin as substrate. Relative activity of the K-As was calculated, in comparison with the activity of K-As without heating.

3.5.5.2 Effect of pH and pH stability on K-As activity

The effect of pH on K-As activity was studied at different pH using 50 mM acetate-phosphate buffer (pH 2.5-7.0). The K-As activity was determined using K-As activity assay conditions (see section 3.5.4).

The pH stability of K-As was determined by pre-incubating the crude K-As with the same buffers, but the concentration was changed to 100 mM. Crude of K-As was mixed with an equal volume of buffer and incubated at 55°C for 1 h. The pH of solution was subsequently adjusted to pH 4.0 and kept at 4°C. K-As activity assay was then carried out using 2% (w/v) gelatin as substrate

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under the optimal pH and using appropriate buffer and the residual activity was determined using K-As activity assay conditions (see section 3.5.4).

3.5.5.3 Effect of salt concentration and salt stability on K-As activity

The effect of salt concentration (NaCl) on K-As activity was determined by using 2% (w/v) gelatin as substrate in 0.05 M sodium acetate buffer (pH 4.0) containing various salt concentrations (0-4 M.). The K-As activity was determined using K-As activity assay conditions (see section 3.5.4).

The effect of salt concentration on K-As stability was determined by pre-incubating the crude K-As in 0.05 M sodium acetate buffer (pH 4.0) containing various salt concentrations (0-4 M.) at 55°C for 1 h. When incubation time was reached, desalt the crude K-As by dialysis method in the same buffer at 4°C for 6 h (4 times for changes new buffer). The K-As activity was determined using K-As activity assay conditions (see section 3.5.4).

3.5.5.4 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The protein patterns of K-As were analyzed by using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE), according to the method of Laemmli (1970). Prior to the analysis, protein contents in crude K-As were evaluated using the Lowry method. The samples were prepared by mixing the crude K-As at a ratio 1:1 (v/v) (15 µg protein) with the sample buffer (0.5 M Tris-HCl, pH 6.8, containing 4% SDS, 20% glycerol and 2 mg/ml bromophenol blue) in the presence of 2% β-mercaptoethanol, representing reducing conditions. Samples in which the heat treatment at 100°C for 3 min was loaded onto polyacrylamide gels comprising (12.5 running gel and a 4% stacking gel and was subjected to electrophoresis at a constant current of 20 mA per gel using an ATTO AE-6530 Dual mini-slab system After electrophoresis, the gel was stained with 0.125% (w/v) Coomassie Brilliant Blue R-250 in 45% (v/v) ethanol and 10% (v/v) acetic acid and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. High molecular weight markers were used to estimate the molecular weight of the proteins.

3.5.5.5 Gelatin zymography

Gelatin zymography was performed according to the method of Wilson *et al.* (1991) with slight modification. The crude K-As (15 µg of protein) was subjected to electrophoresis in a

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final concentration of 0.1% gelatin-containing polyacrylamide (12.5% acrylamide) gel under non-reducing conditions. Samples were electrophoresed at a constant current of 20 mA per gel at 4°C. After electrophoresis, the gel was rinsed twice with distilled water and slowly agitated at room temperature for 1 h in 2.5% (v/v) Triton X-100 (2 changes) in water to remove the SDS. Following this, the gel was rinsed again with distilled water 3-4 times to remove the 2.5% TritonX-100, and then incubated in 0.05 M Na-Acetate buffer (pH 4.0) at 55°C overnight (18-24 h). The gels were fixed and stained for at least 1 h in a 0.1% (w/v) solution of amido black in methanol : acetic acid : water (30 : 10 : 60) and destained in methanol : acetic acid : water (30 : 10 : 60). Areas of proteolysis were detected by observing a clear area against a blue-stained background of undigested gelatin.

3.5.5.6 Protein determination

The protein concentration was determined by the method of Lowry *et al.* (1951). Bovine serum albumin was used as a standard (Appendix C).

3.5.6 Optimization conditions for silver recovery from used x-ray film by K-As

3.5.6.1 Selection of significant variables by Plackett-Burman's design (PBD)

PBD was introduced in this study as a first optimization step to identify which factors have a significant effect on enzymatic hydrolysis for recovery of silver from used x-ray film. Based on PB factorial design, each variable was examined among n variables in $n+1$ experiment in two levels: -1 for low level and +1 for high level, and three center points were run to evaluate the linear and curvature effects of the variables. The variables chosen for the present study were X_1-K-As concentration, X_2-NaCl concentration, X_3-agitation rate, X_4-Time (Table 3.1). Total of four factors were selected for the study with each variable being represented at two levels, high and low, and seven dummy variables in 12 trials (Table 3.2). The range of pH and temperature were fixed on the basis of optimum condition for enzyme activity and that of x-ray films size and weight on the basis of previously report.

X-ray films were washed with distilled water and wiped with cotton impregnated with ethanol. The washed film was dried in an oven at 40°C for 30 min (Shankar *et al.,* 2010). One chip of X-ray film (cut into 2 x 2 cm piece) was then incubated in 20 ml of solution containing the entire combinations of components mentioned in Table 3.2 at 55°C, pH 4. All experiments were carried out

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in triplicate. The weight of X-ray films before and after run was taken as response (Y_1). Visual determination of the films clearly revealed the ability of K-As to strip the gelatin in X-ray films leaving a clean polyester sheet while releasing silver into the hydrolysate.

Table3.1 Variables representing medium solutions used in PBD

Variables	**Unit**	**Coded levels**		
		Low (-1)	**0**	**High (+1)**
X_1: K-As concentration	U/ml reaction	0.05	0.275	0.5
X_2: NaCl concentration	Molar	0	2	4
X_3: Shaking rate	RPM	0	100	200
X_4:Time	Min.	5	17.50	30

Design Expert® version 8.0 Stat-Ease, Inc., Minneapolis, USA, was used to analyze the experimental PBD. A statistical procedure was used to calculate the limit to which the effects of important independent variables are assigned. The significant level (*P*-value) of each main effect was determined using *F*-test. PB experimental design is based on the first-order polynomial model:

$$Y = \beta_0 + \Sigma \beta_1 X_i \quad (4.1)$$

Where: Y is the response (silver content in x-ray film), β_0 is the model intercept

β_1 is the linear coefficient

X_i is the level of the independent variable.

This design does not consider the interaction effects among variables and it was used to screen and evaluate the important variables that influence the response. From the regression analysis of the variables, the significant factors ($p < 0.05$) for silver recovery were further optimized by response surface methodology (RSM).

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Table 3.2 PBD showing four variables with low and high levels coded and values along with the observed results for silver recovery by K-As

Run No.	X_1: K-As Conc. (U/ml reaction)	X_2: NaCl Conc. (M)	X_3: Shaking rate (RPM)	X_4: Time (Min)	Y_1: Loss weight (g)
1	(+1) 0.5	(-1) 0	(+1) 200	(+1) 30	
2	(+1) 0.5	(+1) 4	(-1) 0	(+1) 30	
3	(-1) 0.05	(-1) 0	(+1) 200	(-1) 5	
4	(-1) 0.05	(+1) 4	(-1) 0	(+1) 30	
5	(-1) 0.05	(+1) 4	(+1) 200	(-1) 5	
6	(+1) 0.5	(+1) 4	(+1) 200	(-1) 5	
7	(+1) 0.5	(-1) 0	(+1) 200	(+1) 30	
8	(0) 0.275	(0) 2	(0) 100	(0) 17.5	
9	(-1) 0.05	(-1) 0	(-1) 0	(-1) 5	
10	(0) 0.275	(0) 2	(0) 100	(0) 17.5	
11	(+1) 0.5	(-1) 0	(-1) 0	(-1) 5	
12	(0) 0.275	(0) 2	(0) 100	(0) 17.5	
13	(+1) 0.5	(+1) 4	(-1) 0	(-1) 5	
14	(-1) 0.05	(-1) 0	(-1) 0	(+1) 30	
15	(-1) 0.05	(+1) 4	(+1) 200	(+1) 30	

3.5.6.2 Optimization by Response Surface Methodology (RSM)

The main effect of various factors on silver recovery by K-As has been previously studied using PBD approach. The next step in the optimization for silver recovery was to determine the optimal levels of the significant variables in silver recovery process. The central composite design (CCD) of RSM is one of the most commonly used for fitting second-order models. A CCD has three groups of design points consists of F two-level factorial design points, $2k$ axial points ($\pm\alpha$), and n_c center points. The axial points provide additional levels of the factor for purposes of estimation of the quadratic terms. In this study, the CCD was adopted to optimize the enzymatic hydrolysis for

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recovery of silver from used x-ray film. The most significant variables as determined from PBD, NaCl and enzyme concentration were assessed at five coded levels ($-\alpha$, –1, 0, +1, $+\alpha$). The experiments were designed by Design Expert software. A total of 13 trials were employed with three replicates at the centre point. The visual determination and loss weight of X-ray film were noted and recorded as response, respectively. The coded and actual values of the variables at various levels are given in Table 3.3 and the full experimental plan with respect to their values in actual and coded form is listed in Table 3.4.

X-ray films were washed with distilled water and wiped with cotton impregnated with ethanol. The washed film was dried in an oven at 40°C for 30 min. One chip of X-ray film (cut into 2 x 2 cm piece) was then incubated in 20 ml of solution containing the entire combinations of components mentioned in Table 3.4 at 55°C, pH 4. All experiments were carried out in triplicate. The weight of X-ray films before and after run was taken as response (Y_1). Visual determination of the films clearly revealed the ability of K-As to strip the gelatin in X-ray films leaving a clean polyester sheet while releasing silver into the hydrolysate. The data obtained from the RSM on silver recovery were subjected to analysis of variance (ANOVA). After running the experiments and measuring the loss weight of X-ray film, the experimental results of RSM were fitted with the response surface regression procedure using the following second-order polynomial equation:

$$Y= \beta 0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad (4.2)$$

Where, Y is the response; $\beta 0$ intercept; β_1, β_2, β_3 linear coefficients; β_{11}, β_{22} and β_{33} squared coefficients; β_{12}, β_{13} and β_{23} interaction coefficients.

Table 3.3 Central composite design matrix with coded and actual levels

Variables	Unit	Symbol	Coded levels				
			$-\alpha$	-1	0	+1	$+\alpha$
NaCl	Molar	X_1	- 0.41	0	1.00	2.00	2.41
K-As	U/ml reaction	X_2	- 0.50	0.005	0.0775	0.15	0.18

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Table 3.4 Experimental plan for silver recovery by RSM using CCD

Trial	X_1: NaCl Conc. (M)	X_2: K-As Conc. (U)	Y_1: Loss weight (g)
1	1.00	-α	
2	1.00	0.0775	
3	1.00	0.0775	
4	0.00	0.005	
5	2.41	0.0775	
6	1.00	0.0775	
7	-α	0.0775	
8	1.00	0.18	
9	2.00	0.005	
10	0.00	0.15	
11	2.00	0.15	
12	1.00	0.0775	
13	1.00	0.0775	

The statistical significance of the model equation and the model terms were evaluated via Fisher's test. The quality of fit of the second-order polynomial model equation was expressed via the coefficient of determination, R^2, and the adjusted R^2. The fitted polynomial equation was then expressed as three-dimensional surface plots to illustrate the relationship between the responses and the experimental levels of each of the variables utilized in this study.

3.5.6.3 Validation of the model

The validation of statistical model was done by carrying out the experiment at optimum values of the process parameters (K-As concentration, NaCl concentration, shaking rate, time, pH and temperature) as determined from the model. For this process, the chip of X-ray films (2 x 2 cm) was incubated in 20 ml solution containing 0.15 U/ml reaction, pH 4.0 at 55°C for 5 min in static condition without NaCl. All the experiments were done in triplicate and the experimental values obtained were compared to the values predicted by the model.

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static condition without NaCl. All the experiments were done in triplicate and the experimental values obtained were compared to the values predicted by the model.

3.5.7 Reusability of K-As for silver recovery

Reusability of K-As and solution for gelatin hydrolysis and silver removal was investigated. The X-ray film was incubated in the solution under the optimal condition. After complete removal of gelatin, old X-ray film sheet was removed and fresh film was added to the same solution and incubated for 5 min. The process was repeated until gelatin layer hydrolysis stop was observed. The weight of X-ray film before and after each run was also noted. Visual examination of the film clearly was determined.

3.5.8 Silver recovery

Ten grams of X-ray films was incubated at 55°C, pH 4.0, 0.15 U/ml reaction with and without NaCl in static condition for 5 min. After all silver grains on X-ray films sheet was completely removed from the base films, the clear X-ray films was removed and dried. The weights of X-ray films before and after treated were recorded and analyzed using SEM-EDS. All the silver had been removed from the solution by evaporated and the precipitated was subsequently dried. The precipitate was placed in a crucible and smelted for 3 h at 1000°C to obtain pure silver grains.

3.5.9 Analytical methods

3.5.9.1 Weight loss of films

The weight of X-ray films before and after each run was determined by using a balance.

3.5.9.2 Visual determination

The clarity of X-ray films before and after treatment was determined using a camera.

3.5.9.3 Chemical concentration

The composition of element in X-ray films was performed using an X-ray fluorescence spectrometer (XRF).The liquid specimen was analyzed directly by pouring into sample cup without binder. For the X-ray films sheet, the film was cut into 2 x 2 cm pieces and then, fixed with sample

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holder. The instrumental configuration of the Philips PW 2404 operated at 25±5°C with relatively humidity at 60±10%.

3.5.9.4 Scanning Electron Microscope and Energy Dispersive Spectrometer (SEM-EDS)

The morphological characterization of X-ray films was performed using SEM-EDS. The X-ray films were cut into 1x1 cm without a thin gold layer coating. Pre-selected areas of the sample surface were scanned with a focused electron beam using a JEOL JSM-5410LV scanning electron microscope (SEM) with low vacuum mode at 30 Pa. The electron beam interacts with the sample surface producing backscattered electrons and characteristic X-rays depending on the chemical composition of the sample surface. Heavier elements generate more back scattered electrons, so the brightness in the image corresponds to its chemical composition. The difference in the brightness level in the image is used to separate the heavier inorganic particles from the dark organic matrix using image processing software. The elemental composition can be determined with a Link ISIS energy dispersive X-ray spectrometer (EDS). The acceleration voltage of 20 kV was used in the SEM–EDS analyses. The EDS detector was capable of detecting elements with atomic number equal to or greater than six. The intensity of the peaks in the EDS is not a quantitative measure of elemental concentration, although relative amounts can be inferred from relative peak heights.

3.5.10 Statistical analysis

All experiments were done in triplicate. A completely randomized design was used throughout this study. Data was subjected to analysis of variance and mean comparison was carried out using Duncan's Multiple Range Test. Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for Windows: SPSS Inc.)

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CHAPTER IV

RESULTS AND DISCUSSION

4.1 Characterizations of Kumamolisin-As

4.1.1 Optimum temperature and thermal stability

The effect of temperature on the K-As activity was analyzed using various temperatures ranging from 37°C to 80°C at pH 4.0 as shown in Fig. 4.1A. From 37°C, K-As activity significantly increased and reached a maximum activity at 55°C. No significant difference was found in relative enzyme activity from 55°C to 60°C, with almost 95% of highest activity retained. However, a sharp decline was observed after incubation at 70°C. The result indicates that K-As has activity at wide temperature range. Similar results of optimum activity at elevated temperatures have been shown in some kumamolisins described previously and were lower than those of Kumamolisin-Ac from *Alicyclobacillus acidocaldarius*, which had optimal temperature at 60°C. Murao *et al.* (1993; 1998) and Narutaki *et al.* (1999) reported that thermostable proteinase, Kumamolisin from *Bacillus* sp. strain MN-32 were optimally active at 70°C for 30 min using casein as a substrate, and lost its activity at 90°C for 10 min. Okamoto *et al.* (2001) also reported that a novel collagenolytic serine-proteinase of *Bacillus* sp. strain MO-1 was active at 60°C for 30 min.

According to the current results, K-As showed decreased activity at 70°C. The inactivation at high temperature is possibly due to the partial unfolding of the enzyme. Generally, activity of microbial carboxyl proteinase was inactivated about 50% after incubation at 60 to 65°C for 15 min (Murao *et al.,* 1993). Because, in the current study, K-As showed the maximum activity at 55°C; therefore, 55°C was selected for subsequent determinations.

Thermal stability of K-As was also examined by determining the residual activity of the enzyme after incubation at various temperatures (37-80°C) for 1 h (Fig. 4.1B). It must be noted that the residual activity was determined at temperature at which the enzyme showed maximum activity (55°C).

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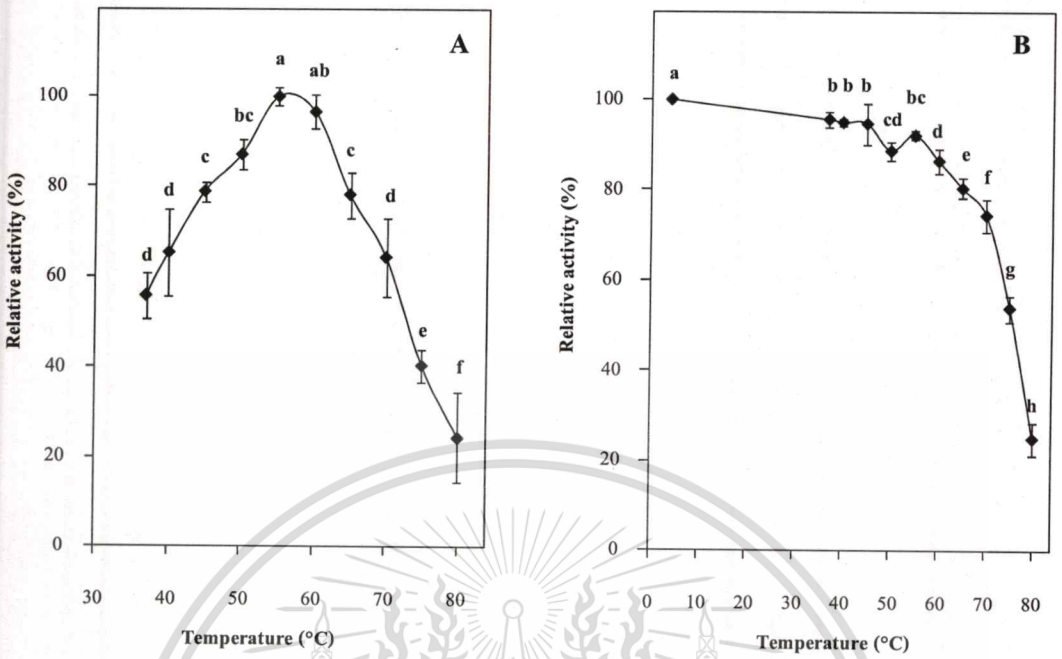

Figure 4.1 Temperature profile (A) and thermal stability (B) of K-As activity. The activity was measured using gelatin as substrate in 0.05 M Na-Acetate buffer pH 4.0 for 1 h at various temperatures (A). For the stability test, the K-As was incubated for 1 h at the temperatures indicated in 0.05 M Na-Acetate buffer pH 4.0, and then cooled on ice. Residual activity was measured using gelatin as substrate at 55°C with pH 4.0 for 1 h. The different letters in the same line denote the significant differences (p<0.05). Average ±standard deviation from a triplicate determination.

The heat stability profile showed that K-As remained completely active at a broad range of temperature from 37 to 70°C. However, the activity rapidly decreased as the reaction temperature increased above 70°C. The thermal stability of K-As found in this study corresponded with the stability of other Kumamolisins previously reported. Kumamolisin from *Bacillus* MN-32 was stable at 50°C after heated for 24 h (Murao *et al.*, 1988). Such stability was also observed for Kumamolisin-ac from *Alicyclobacillus acidocaldarius* at 80°C for 10 h (Catara *et al.,* 2006). However, collagenolytic thermostable from *Bacillus* sp. strain MO-1 is active after incubation at 60°C for 30 min.

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4.1.2 Optimum pH and pH stability

The effect of pH on K-As activity was studied by determining activity of the enzyme at various pH values using gelatin as a substrate. Data presented in Fig.4.2A indicate that the K-As was active over a wide range of pH from 2.5 to 5.5 recording maximal activity at pH 4.0 compared to that noted at other pH values. Enzyme activity slowly decreased under pH above 4.5. The results in this study are in agreement with earlier reports showing pH optima at acidic condition for Kumamolisin and other serine peptidase (Murao *et al.,* 1993; 1998 and Narutaki *et al.,* 1999; Catara *et al.,* 2006). In 1993 and 1998, Murao and colleagues reported the optimal pH of a Kumamolisin from *Bacillus* novo sp. strain MN-32 was around 3.0. Similarly, Catara and co workers (2006) reported that K-ac from *A. acidocaldarius* was active at extremely acidic condition (pH 1.0-4.0), showing maximal activity at pH 2.0. The optimum activity of collagenase from *Bacillus* sp. MO-1 was observed at pH 7.5 (Okamoto *et el.,* 2001), while the collagenase of *Streptomyces parvulus* subsp. *citrinus* and *Clostridium histolyticum* were completely after incubation at 60°C pH 7.5 (Nakayama *et al.,* 2000). A recent report of Oda *et al.* (2012) shows that the optimum pHs of serine carboxyl peptidases, PSCP from *Pseudomonas* sp., XSCP from *Xanthomonas* sp., Kumamolisin-B (J-4) from *Bacillus coagulans*, and TPP-1(CLN2) from *Homo sapiens* were around pH 2.7 to 3.5. Furthermore, it was observed that the K-As activity loss virtually exhibited at neutral and highly alkaline pH conditions indicating that the reaction of enzyme can be easily stopped by shifting the pH to 7.0. The result is supported by the study of Tsuruoka *et al.* (2003).

The pH stability of the enzyme was assayed by measuring the residual activity at various pHs (pH 2.5 to 7.0) after 1 h of incubation at 55°C. Fig. 4.2B shows that the K-As is stable over a wide range of acidic pH (2.5-5.5) in which K-As retained more than 55% of its maximum activity.

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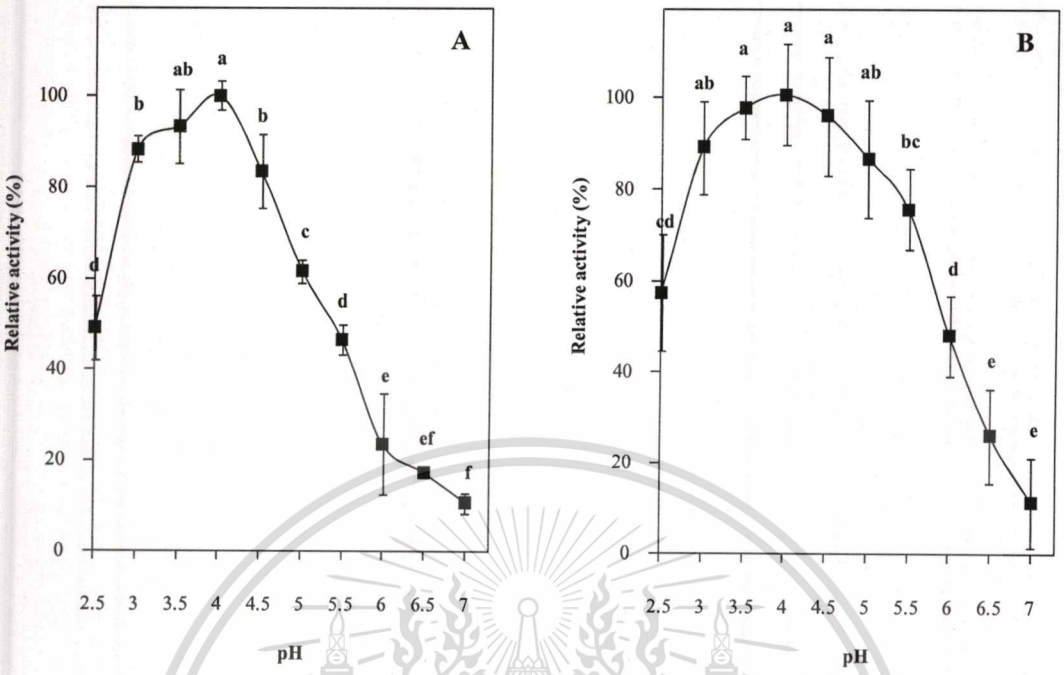

Figure 4.2 Effect of pH (A) and pH stability (B) of K-As activity. The activity was measured using gelatin as substrate for 1 h in 0.05 M phosphate-citrate buffer at 55°C with various pHs. For the pH stability test, the K-As was mixed with 0.1 M phosphate-citrate buffer in an equal volume and incubated at 55°C for 1 h at various pHs. Residual activity was measured using gelatin as substrate at 55°C with pH 4.0 for 1 h. The different letters in the same line denote the significant differences ($p<0.05$). Average ±standard deviation from a triplicate determination.

In addition, K-As is highly stable at pH 4.0 with the activity remained 100% after incubation at 55°C for 1 h when compared with initial activity. Similar results were shown by some collagenases described previously and were higher than those of K-As, of the genera *Bacillus* (Nakayama *et al.*, 2000) and of acid proteinase from thermophilic *Bacillus* novo sp. strain MN-32, which had stable more than 80% at the pH 4.0, 60°C for 4 h and 100% at pH 2-5 in 24 h, respectively (Murao *et al.,* 1988). The pH stability of K-As was similar to that of K-ac when the enzyme was remarkably stable with a half life of 10 h at 80°C, pH 2.0 (Catara *et al.*, 2006).

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4.1.3 Optimum salt and salt stability

The effect of salt on K-As activity was studied by adding NaCl into the reaction mixture at the concentrations ranging from 0 to 4.0 M. Activity of the enzyme was determined at pH 4.0 and 55°C using gelatin as a substrate. The enzyme also showed activity at relatively higher NaCl concentration compared to the reaction mixture without salt. A gradual increase in K-As activity was observed with increasing NaCl concentration from 0 to 4.0 M and the enzyme showed the highest activity at 4 M NaCl (Fig.4.3A). Interestingly, the enzyme showed high activity in the presence of salt at 1.5- 4.0 M. No significant difference was found in relative enzyme activity from 1.5 to 4 M NaCl, with almost 85 % of K-As remained active. The addition of NaCl resulted in an activation of K-As around 30% of its initial activity. These results indicate that NaCl was very effective in improving the activity of the enzyme. Such results are in line with those of Kumamolisin-ac, Catara *et al.* (2006) reported that the addition of 5 mM $CaCl_2$ in the reaction mixture gave rise to a 1.6 fold increase in activity of K-ac. Therefore, 4 M NaCl was used in subsequent determinations in the current study.

The salt stability of K-As was assayed by measuring the residual activity after incubation at various NaCl concentrations at 55°C for 1 h. After salt was removed, the enzyme stability was tested at optimum salt concentration (4 M), pH 4.0 and 55°C for 1 h. The salt stability profile (Fig 4.3B) shows that K-As remained active at a wide range of salt concentration. The enzyme was found to be stable in the presence of salt as it retained about 90% of its activity in all concentrations tested after 1 h incubation. The stability of K-As in the presence of high salt concentrations revealed that it is highly halotolerant enzyme besides being pH and temperature stable.

The observed characteristics and stability of Kumamolisin-As revealed several potential practical advantages of K-As in the several industrial processes. Moreover, K-As was active the most under thermoacidophilic and high salt concentration conditions (at 55°C pH 4.0 and NaCl 4 M), which can effectively eliminate the possibility of microbial contamination of the reaction system. For example, the observed characteristics of K-As should allow the silver recovery and the efficient enzymatic production of gelatin hydrolysate from used X-ray films. This study highlights the suitability of this enzyme for applications in industrial processes carried out at acidic, high temperature and a combination with or without salt.

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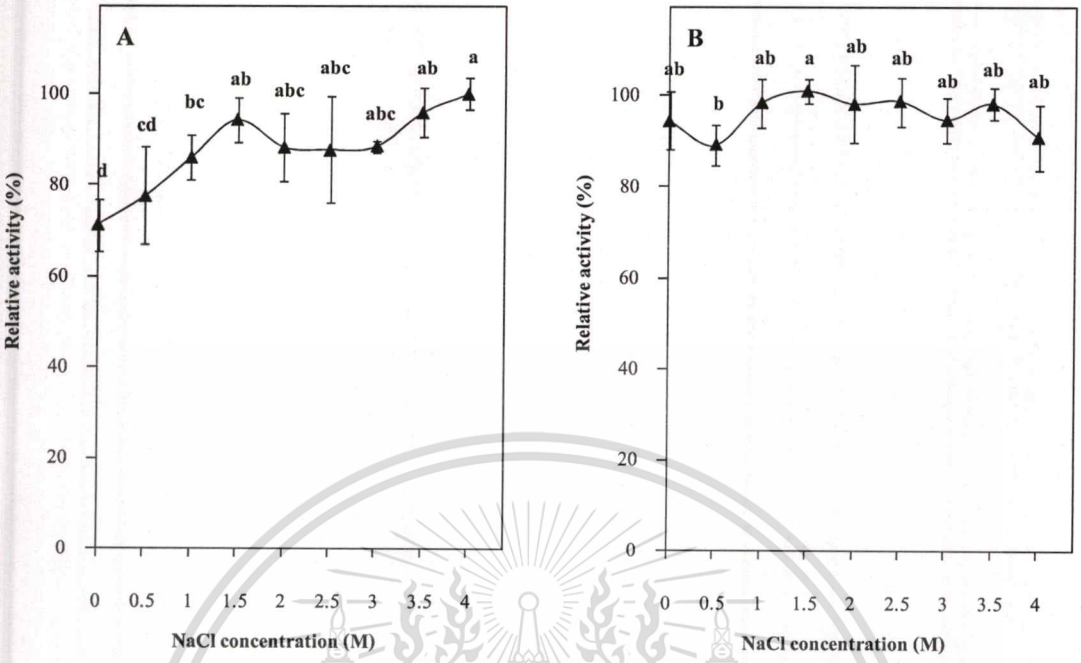

Figure 4.3 Effect of NaCl (A) and NaCl stability (B) profile of K-As activity. The K-As activity was analyzed using gelatin as substrate for 1 h at pH 4.0 and 55°C with various salt concentrations. For the stability test, the enzyme was incubated at 55°C for 1 h in 0.05 M Na-acetate buffer (pH 4.0) containing various NaCl concentrations. Residual activity was analyzed using gelatin as substrate for 1 h, pH 4.0 in the presence of 4 M NaCl at 55°C. Bars represent the standard deviation (n=3). The different letters in the same line denote the significant differences (P<0.05).

4.1.4 Protein pattern and gelatin zymography

The enzyme was partially purified by acid treatment at 55°C and pH 4.0 for 5 h. The protein pattern of K-As was determined by SDS-PAGE and gelatin zymography by using the conditions of Wilson *et al.*, 1991 with slight modifications. The results revealed two activity bands corresponding to molecular masses of 41 and 45 kDa from both SDS-PAGE (lane 1, Fig. 4.4) and zymography (lane 2, Fig. 4.4).

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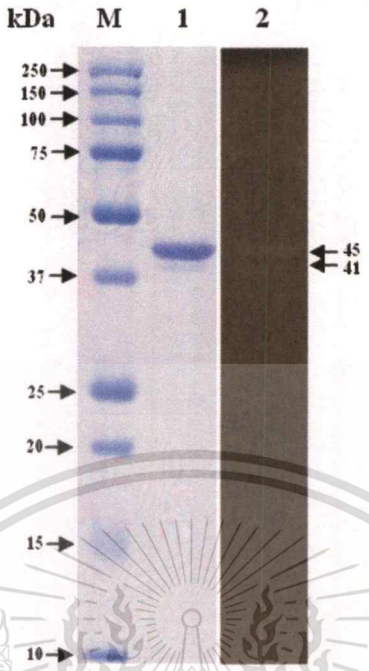

Figure 4.4 SDS-PAGE and gelatin zymography of partial purified Kumamolisin-As (after incubation at 55°C and pH 4.0 for 5 h). Lane M, broad range protein molecular marker, lane 1, protein pattern performed by using the conditions of Laemmli *et al.* (1987), lane 2, gelatinolytic activity using gelatin as substrate (0.1% w/v) by the modification conditions of Wilson *et al.* (1991) and stained by silver staining

Oda (2012) reported that the serine carboxyl-peptidase groups have a large amino-terminal pro-region composed of around 200 amino acid residues and the molecular mass is about 40 kDa. Tsuroka and co workers (2003) showed that K-As is expressed as a 57 kDa precursor. Under acidic conditions, K-As is autocatalytically converted into the active 46 kDa which is significantly larger than the *m/z* value obtained from the MALDI-TOF (MS) analysis of the gel purified enzyme (*m/z* 37.100±450). Due to the relatively high contents of acidic amino acid residues in the enzyme, as suggested by Zirwes *et al.* (1997), the irregularly low electrophoretic mobility of K-As in SDS-PAGE can be expected. Similar observations are also reported for Kumamolysin from *Homo sapiens* (Oyama *et al.,* 2002); K-As (Tsuruoka *et al.,* 2003) and scytalidopepsin B (Shimuta *et al.,* 2000).

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4.2 Application of K-As in silver recovery

4.2.1 Screening of significant variables using Plackett-Burman design (PBD)

A PB design is a well-established and widely used statistical procedure for screening variable factors that potentially show statistical significance in this bioprocess. Each analyzed variable factor was evaluated at three levels, high level (+1), center point (0) and low level (-1). In this study, four variables were investigated including K-As concentration, NaCl concentration, shaking rate and hydrolysis time. Treatment ranges of each factor were selected based on prior knowledge obtained from literature reviews the software generated 15 experimental runs regarding the effects of the factors on responses, including loss weight of film (Table 4.1) and visual determination.

Subsequently, statistical analysis of the model was performed, and the variables evidencing a statistical significance were screened via Student's t-test for ANOVA (Table 4.2). The results showed that the model is significant (F value of 8.13) and there is only a 0.35 percent chance that a "Model F-Value" this large could occur due to noise. Confidence of the predicted model can be demonstrated by a multiple correlation coefficients (R^2 value). The R^2 value is always displayed between 0 and 1. The closer the R^2 value to 1, the more the excellent the correlation between the observed and predicted values is. The model with R^2 value > 0.75 is still acceptable. In this study, our model showed R^2 = 0.7648, indicating that the model could explain up to 76.48 % variation of the data achieved. Therefore, the model was appropriate and the investigated factors were assessed for significance and contribution. The variable factors were screened at confidence level of 95% based on their effects. Also, factors evidencing p-values less than 0.05 were considered to have significant effects on the response, and should be selected for further optimization.

The resulting equation can be used to predict a response that occurs with a variation of the factors included therein.

$$Y = +3.12389 - 5.55556 \times \text{Enzyme Concentration} - 4.20833 \times \text{NaCl Concentration} - 3.08333 \times \text{Shaking rate} + 4.66667 \times \text{Time} \quad (4.1)$$

where Y is the response variable (loss weight of X-ray films)

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In the case of shaking rate, a change to the higher level led to a decrease in the loss weight of X-ray films. The confidence level of the factor with negative effect (shaking rate) was 92.11 %, much lower than the predefined level of significance and hence, was not considered for further optimization. For K-As concentration, high concentration of K-As in process manipulation caused an increase in the loss weight of X-ray films. In the case of the hydrolysis time, a change to the lower level lead to an increase in the loss weight of X-ray films and showed a high contribution to silver recovery but it was not significant when compared to the other variables. When focusing on NaCl concentration, the results showed that the experiment in the presence of NaCl gave the low value in weight loss of X-ray films. The loss weight of X-ray films decreased with increasing concentration of NaCl. An increase in the loss weight of X- ray films may be due to a change in the NaCl/K-As ratio. It can be explained that when silver is released from the film, silver undergoes interacting with NaCl, resulting in AgCl. Although effect of NaCl concentration tested in the present study had a significant but it showed negative effect on silver recovery process. Furthermore, NaCl shows a high contribution (67.10 %) to silver recovery when compared to the other variables. Additionally, characterizations of K-As indicated that silver removal might be depended on the interactions between salt and enzyme concentration. Therefore, NaCl concentration, with a p-value of 0.0003, was determined to be the most significant factor for silver recovery. The rest of the factors such as concentration of K-As, shaking rate and time for hydrolysis showed confidence level below 90% and hence, were considered insignificant.

In this study, we aimed to develop the silver recovery process, which is eco-friendly and low cost. The high cost of process is typically from preparation step of the enzyme. Based on Plackett–Burman screening, concentration of NaCl and concentration of K-As were selected for further optimization by RSM using CCD. The other variables shown insignificant effect would be maintained at constant level as well as pH at 4.0 and temperature at 55°C.

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Table 4.1 Response of PBD studies for silver recovery by K-As

Run No.	K-As Conc. (U/ml Rxn)	NaCl Conc. (M)	Shaking rate (RPM)	Time (min)	Loss weight of X-ray films (g)
1	0.5	0	200	30	0.0027
2	0.5	4	0	5	0.0017
3	0.275	2	100	17.50	0.0033
4	0.05	0	200	5	0.0020
5	0.275	2	100	17.50	0.0027
6	0.275	2	100	17.50	0.0027
7	0.5	0	0	5	0.0031
8	0.05	0	0	30	0.0032
9	0.05	0	0	5	0.0034
10	0.5	0	200	30	0.0028
11	0.5	4	0	30	0.0011
12	0.5	4	200	5	0.0006
13	0.05	4	200	5	0.0010
14	0.05	4	200	30	0.0012
15	0.05	4	0	30	0.0015

The magnitude of the variable effects on silver recovery are provided by statistical analysis, where the effect of each factor is the difference of the response related with the change of the lower level (-1) to the higher level (+1). When an effect has a great significance level, the process will present significantly better results for one of these levels. Otherwise, if the factor is irrelevant to the process, no change in the process performance takes place. Figure 4.5 represents the effects of each variable in the levels of film weight after being treated with K-As. The negative values indicate that a change of the lower level to the higher level in the corresponding variable low level of loss weight of film after treatment, while the positive values reflect an increase in the loss weight of film due to the change for a higher level. The effects of each variable show a clear trend in its contribution to the response.

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Table 4.2 Analysis of variance (ANOVA) for PBD

Source	Sum of Squares	df	Mean square	F-value	*p*-value Prob>F
Model	9.690	4	2.423	8.13	0.0035
A-K-As Conc.	7.500	1	7.500	0.025	0.8771
B-NaCl Conc.	8.501	1	8.501	28.53	0.0003
C-Shaking rate	1.141	1	1.141	3.83	0.0789
D-Time	4.083	1	4.083	0.14	0.7189
Residual	2.979	10	2.979		
Cor. Total	1.276	14			

(R^2 = 76.48 %; Adj R^2 = 67.08 %; C.V. 24.74 %)

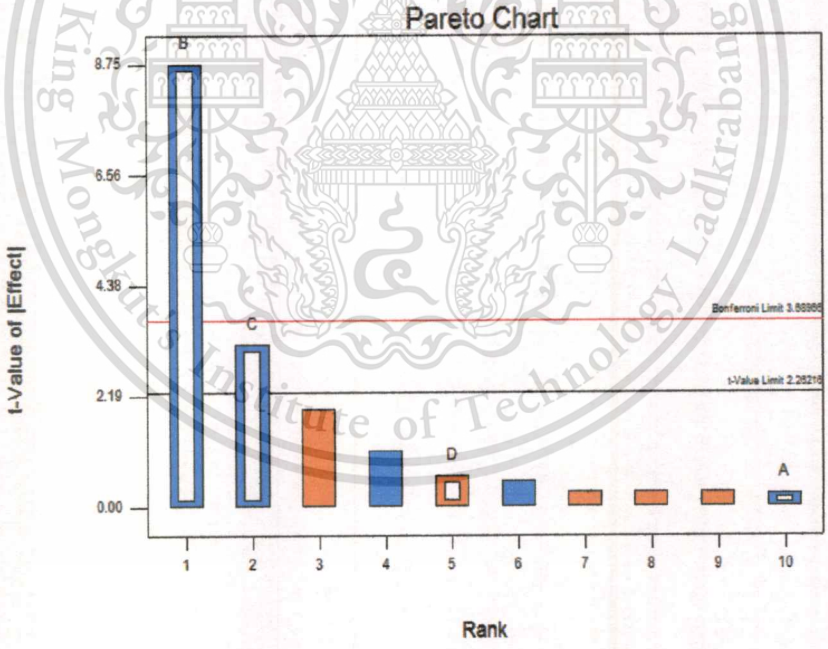

Figure 4.5 Pareto chart showing effects of the variables according to this magnitude based on the observation of PBD. A: K-As concentration; B: NaCl concentration: C: shaking rate and D: time. Blue and orange bar represent in negative and positive effect, respectively.

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Figure 4.6 represents visual examination of X-ray films after treated under the condition from PBD. As it can be seen in the figure, the control film without treated with K-As till unchanged. After treated with K-As, a clean bluish base film was obtained. The silver recovered and impurities in X-ray films were determined by XRF and hence the silver content of X-ray films was calculated to be 0.83% (w/w). Table 4.3 shows the trace metal impurities after recovered silver for 15 metals are content in X-ray films. The film after treated with K-As contained Na, Mg, Al, Si, P, S, Cl, Ca, K, Cr, Fe, Cu, Zn, Sb, I. These findings are in accordance with earlier report of Nakiboglu *et al.* (2001). The authors showed 11 metal impurities, which are Al, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb and Sn as detected by ICP-MS in the process of silver recovery using alkaline protease from *Bacillus subtilis* ATCC6633. In study of Nakiboglu and co-workers (2003), the same impurities were obtained from silver recovery from photographic films using NaOH stripping. After treatment with K-As, 100% silver recovery was achieved with undetected silver content in X-ray films.

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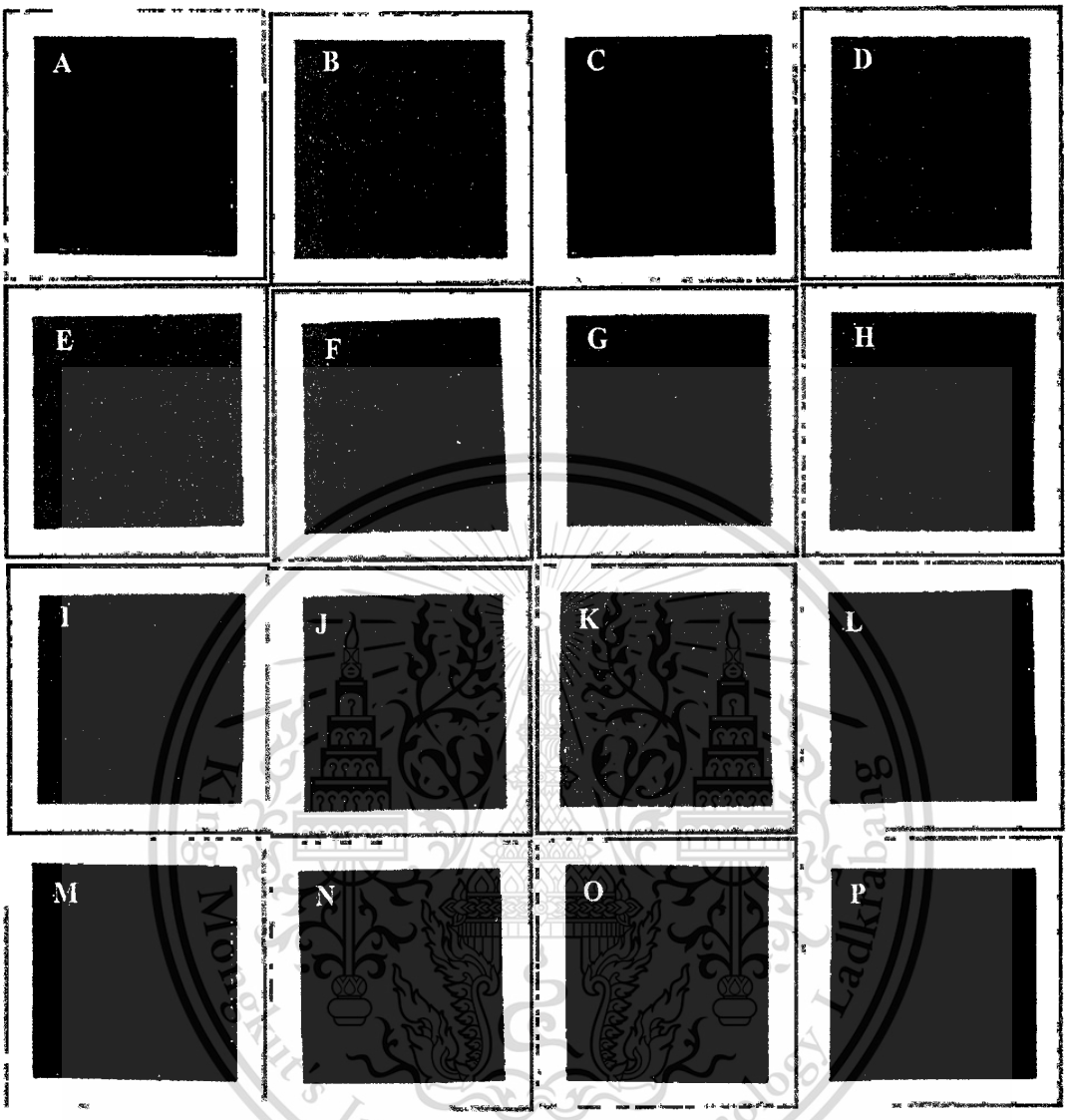

Figure 4.6 Visual examination of the clearly films revealed the ability of K-As to strip the gelatin in X-ray films leaving a clean polyester sheet while releasing silver into the hydrolysate. A: used X-ray films before treatment with K-As as a control and B-P) X-ray films sheet after treatment with K-As under the condition generated by PBD from run 1 to 15.

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Table 4.3 Results of trace impurities in X-ray films (all units in % w/w)

Element	Compound name	Before treatment	After treatment
$C_{10}H_8O_4$	Polyethylene terephthalate (PET)	96.09	99.31
Na	Sodium	0.016	0.017
Mg	Magnesium	0.016	0.01
Al	Aluminium	0.63	0.004
Si	Silicon	1.326	0.01
P	Phosphorus	0.034	0.009
S	Sulphur	0.374	0
Cl	Chlorine	0.532	0.538
Ca	Calcium	0.011	0.005
Ti	Titanium	0.002	0
Cr	Chromium	0.007	0.005
Fe	Iron	0.012	0.015
Cu	Copper	0.005	0.004
Zn	Zinc	0.006	0.008
Ag	Silver	0.827	0
Sb	Antimony	0.059	0.057
I	Iodine	0.057	0
K	Potassium	0	0.003

*Determined by XRF method

4.2.2 Optimization by Response surface methodology (RSM)

In order to search for the optimum condition of the process, CCD with two factors, concentration of the enzyme and NaCl, was conducted with the conditions indicated Table 4.4. It was employed to fit a second order polynomial model. The centre point in the design was repeated five times for determining the error. The experimental design (coded and value) and corresponding

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response are shown in Table 4.4. The experimental results of CCD were fitted into a quadratic polynomial equation as follows:

Final equation in terms of coded factors:

$$Y = +1.240E\text{-}003+1.163E\text{-}003*A -5.169E\text{-}004*B +5.000E\text{-}005*A*B +2.425E\text{-}004*A^2+6.425E\text{-}004*B^2 \quad (4.2)$$

Final equation in terms of actual factors:

$$Y = +1.48716E\text{-}003+4.09796E\text{-}004*\text{K-As conc.} -1.85539E\text{-}003*\text{NaCl conc.} +3.44828\text{-}005*\text{K-As conc.}*\text{NaCl conc.} +1.15339E\text{-}004*\text{K-As conc.}^2 +6.42500E\text{-}004*\text{NaCl}^2 \quad (4.3)$$

where Y is the response variable (loss weight of X-ray films) and A and B are the concentration of K-As and NaCl, respectively.

The polynomial equations comprise the coefficients for intercept, first order main effects, interaction terms, and higher order effects. The sign and magnitude of the main effects signify the relative influence of each factor on the response, i.e., average result of changing one factor at a time from its low to high value. For each run, the experimental responses, along with the predicted response obtained from the regression equation. The experimental results were fitted into second-order response surface model. The polynomial models for response can be represented by the Eq. (4.2), Eq. (4.3), respectively. The ANOVA for the model was performed and is summarized in Table 4.5.

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Table 4.4 Central composite design matrix with experimental and predicted values of weigh loss of X-ray films

Trial	Variables/levels				Loss weight of X-ray films (g)
	K-As (U/ ml Rxn)		NaCl (M)		
	Coded	Actual	Coded	Actual	
1	-α	0	0.0	1.00	0.0007
2	0.0	0.0775	0.0	1.00	0.0012
3	0.0	0.0775	0.0	1.00	0.0016
4	-1.0	0.005	-1.0	0.00	0.0013
5	0.0	0.0775	+α	2.41	0.0019
6	0.0	0.0775	0.0	1.00	0.0014
7	0.0	0.0775	-α	0.00	0.0031
8	+α	0.18	0.0	1.00	0.0029
9	-1.0	0.005	+1.0	2.00	0.0009
10	1.0	0.15	-1.0	0.00	0.0038
11	0.10	0.15	+1.0	2.00	0.0036
12	0.0	0.0775	0.0	1.00	0.0011
13	0.0	0.0775	0.0	1.00	0.0013

The predicted values were compared with the obtained values from the experiment; it indicated that these data were in reasonably close agreement. The fit of the model was checked by the coefficient of determination *R*-square, which was calculated to be 0.8809, indicating that 88.09% of the variability in the response could be explained by the model. The interaction terms (AB) show how the response changes when two factors were simultaneously changed. The second-degree term (A^2, B^2) are included to investigate non-linearity. A negative sign signifies antagonistic effect while a positive sign indicates a synergistic effect. The Model F-value of 10.35 implies that the model is significant. There is only a 0.39% chance that the model could occur due to noise. The "Lack of Fit F-value" of 7.36 implies that the lack of fit is significant. There is only a 4.18% chance that the model could occur due to noise. The value of predicted R^2 (0.2543) is in reasonable agreement with the

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adjusted R^2 (79.58%) also indicated the efficacy of the model. Adequate precision ratio of 9.724 indicates an adequate signal. Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable and the model can be used to navigate the design space. The model was found to be adequate for prediction within the range of variables employed. This ensured a satisfactory adjustment of the quadratic model to the experimental data. The coefficient of variation (CV) indicates the degree of precision with which the treatments are compared. A lower CV means a higher reliability of the experiment. The value of CV (31.16%) demonstrated the performed experiments were reliable. The *P*-values are also used as a tool to check the significance of each coefficient. Smaller value of *P* indicates that the corresponding coefficient is more significant. Values of the probability less than 0.05 indicate that the model terms are significant. In this case A, B and B^2 were the significant model terms. Values greater than 0.05 indicate the model terms are not significant which are AB, A^2 and B^2.

Table 4.5 Regression coefficients and their significance for response surface quadratic model

Source	Ssq	DF	SE	95% CI low	95% CI high	F-value	P-value
Model	1.601	5				10.35	0.0039
A: K-As	1.081	1	2.487	6.519	1.828	34.96	0.0006
B: NaCl	2.138	1	1.966	6.977	1.628	6.91	0.0340
AB	1.000	1	1.966	-9.819	-5199	0.032	0.8624
A^2	4.091	1	2.781	-6.075	7.411	1.32	0.2879
B^2	2.872	1	2.109	-2.561	1..141	9.28	0.0187
residual	2.165	7					
Lack of fit	1.833	3				7.36	0.0418
Pure error	3.320	4					
Cor. total	1.818	12					

R^2 = 88.09% (0.8809); adj R^2 = 79.58% (0.7958); C.V. 31.16%; adequate precision ratio 9.724. *AB* represents the interaction effect of variables *A* and *B*; A^2 and B^2 are the squared effects of the variables. Values in boldface represent significant factors ($p < 0.05$).

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As described above, the correlation between the experimental and predicted values of silver recovery, wherein, the points cluster around the diagonal does not line, which indicated the lack of fit of the model, since the deviation between the experimental and predicted values was high (Figure 4.7). Three-dimensional response plot shown in Fig. 4.8 depicts the behavior of weight loss of X-ray films, main effect, interaction effect and squared effect (nonlinear) of two factors, K-As and NaCl at difference concentrations. An increase in K-As concentration showed a greater weight loss of the film; similarly, increase in NaCl concentration also showed an increased weight loss of X-ray films but not at the level observed in without NaCl. Additionally, the interaction effect of both the concentration level was not found to be significant (*P*-value of AB = 0.8624), implying that the combined factors were not essential for silver recovery from X-ray films. Visual examination of the clear films revealed the ability of K-As to strip the gelatin in X-ray films leaving a clean polyester sheet while releasing silver into the hydrolysate (Fig. 4.9). From the study of response surface 3D plot, the optimum concentration of K-As and NaCl (Fig. 4.8) were found to be 0.15 U/ml reaction and without salt, respectively.

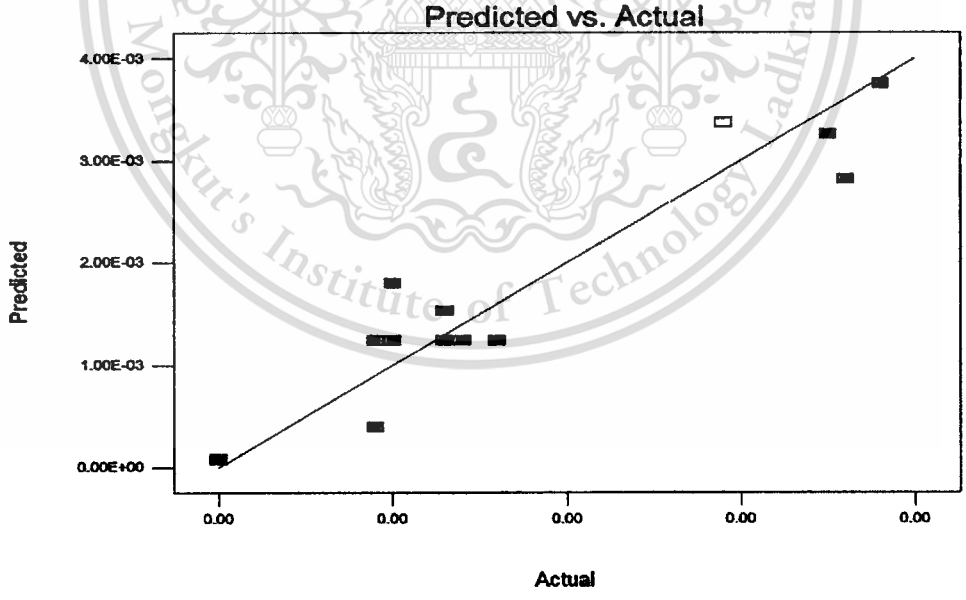

Figure 4.7 Plot of predicted versus actual weight loss of X-ray films (g). Blue-green and red point represents low and high values, respectively.

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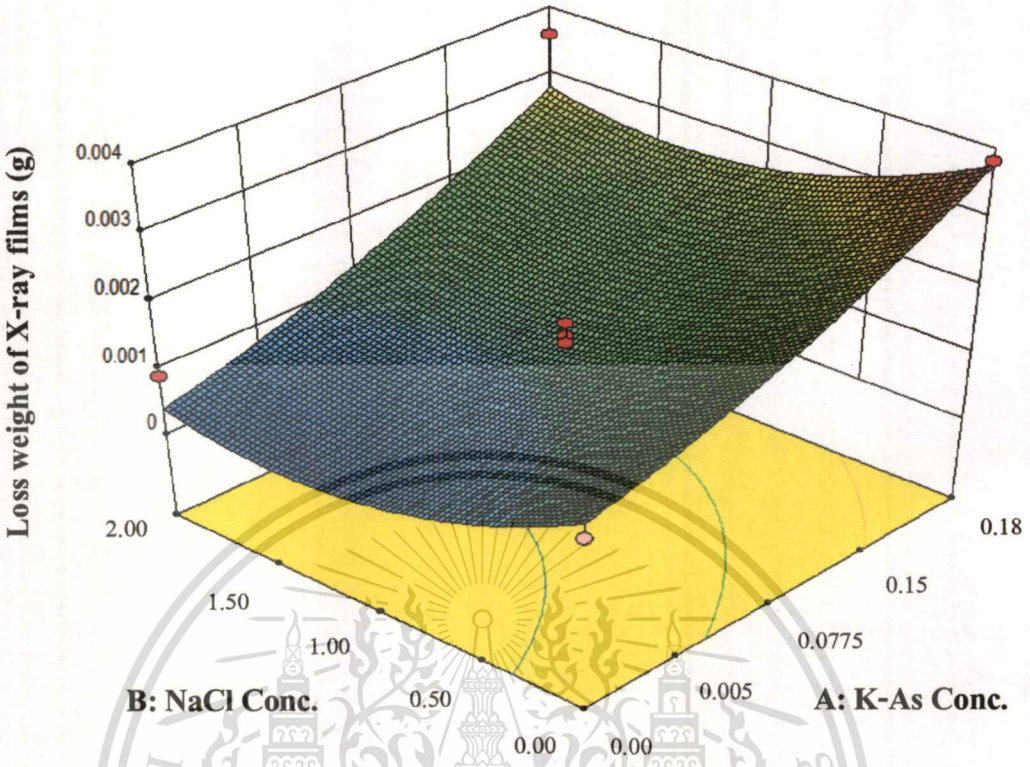

Figure 4.8 Response surface 3D contour plots showing the effect of: (A) K-As concentration and (B) NaCl concentration on silver recovery. Dark-Blue and red point represents low and high values, respectively.

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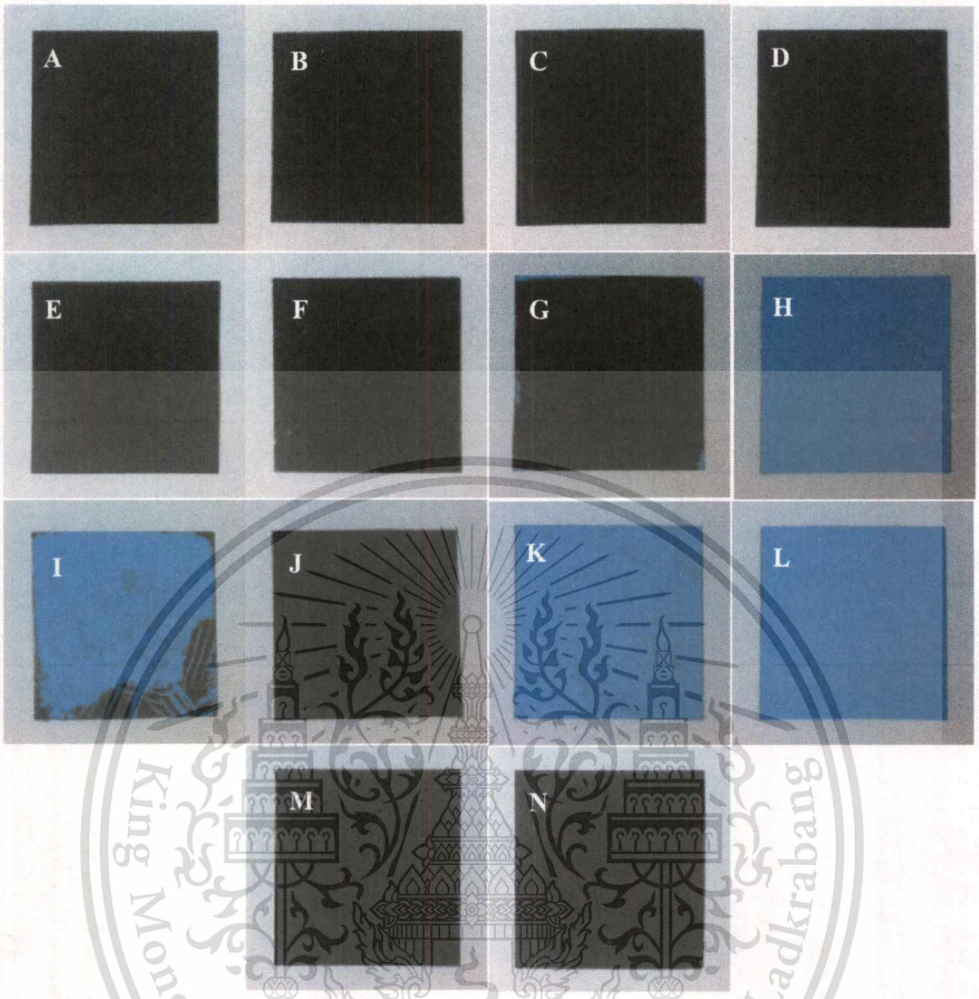

Figure 4.9 A) used X-ray films before treatment with K-As as a control and, B-N) X-ray films sheets after treatment with K-As under the condition generated from RSM (run 1 to 13)

4.2.3 Validation of the experimental model

To validate the optimal operational parameters, confirmatory experiments were carried out. The optimal operational parameters obtained by Design-Expert software were as follows: pH 4.0, concentrations of K-As at 0.15 U/ml of total volume reaction and without NaCl and the temperature of 55°C with static condition for 5 min. Three replicated runs at optimal conditions were carried out to verify the accuracy of the experimental model. Under these optimized conditions, the clear bluish films and 0% (w/w) of silver in X-ray films sheet were determined by visual determination (Fig.

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4.10). These results confirmed the validity of the model and the experimental model was a good fit for the system. Therefore, the study was successful in optimizing the condition for silver recovery from used X-ray films by K-As using Plackett-Burman design and response surface method. Besides, this research has laid down an experimental foundation for practical application.

In order to visualize the existence of morphological changes as a result of K-As degelatinization, the morphological characterization of X-ray films was performed using Scanning Electron Microscope and Energy Dispersive Spectrometer (SEM-EDS). Fig. 4.11 shows the spectrum of the elements in X-ray films. The element mapping, with the carbon, silver, oxygen, sulfur, chloride, silicon, aluminum, iodine and argon over the entire area of the X-ray films was shown in Figure 4.12. This suggests that the distribution of silver on X-ray films is homogeneous. A comparative compilation of scanning electron micrographs of the untreated and treated X-ray films are depicts in Fig 4.13. From Fig 4.13 A, it is apparent that the surface structure of new X-ray films was rough due to silver bromide crystal and gelatin. A cross-section of X-ray films shows a double-emulsion film containing of silver bromide grain, transparent PET film and protective coating on both surfaces (Fig 4.13B). There was little visible structural change on the surface of the untreated with K-As (Fig 4.13C-D). However, after exposure to K-As, X-ray films has undergone obvious changes and reveal extensive smooth surface. Structural changes such as, adhesive, emulsion and protective coating layer disappear from both surfaces were observed in K-As treated. Silver crystal and gelatin missing from the X-ray films surface are evident (Fig 4.13E-F). Thus, K-As induce changes in the morphology and can be useful in silver recovery from X-ray films.

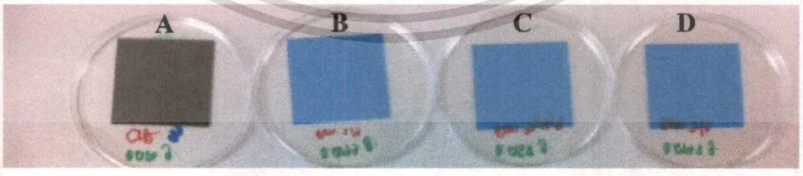

Figure 4.10 X-ray films sheet obtained after enzyme treatment of X-ray films under the optimum condition obtained from RSM (triplicate; B-C) while control film (A) remained unchanged

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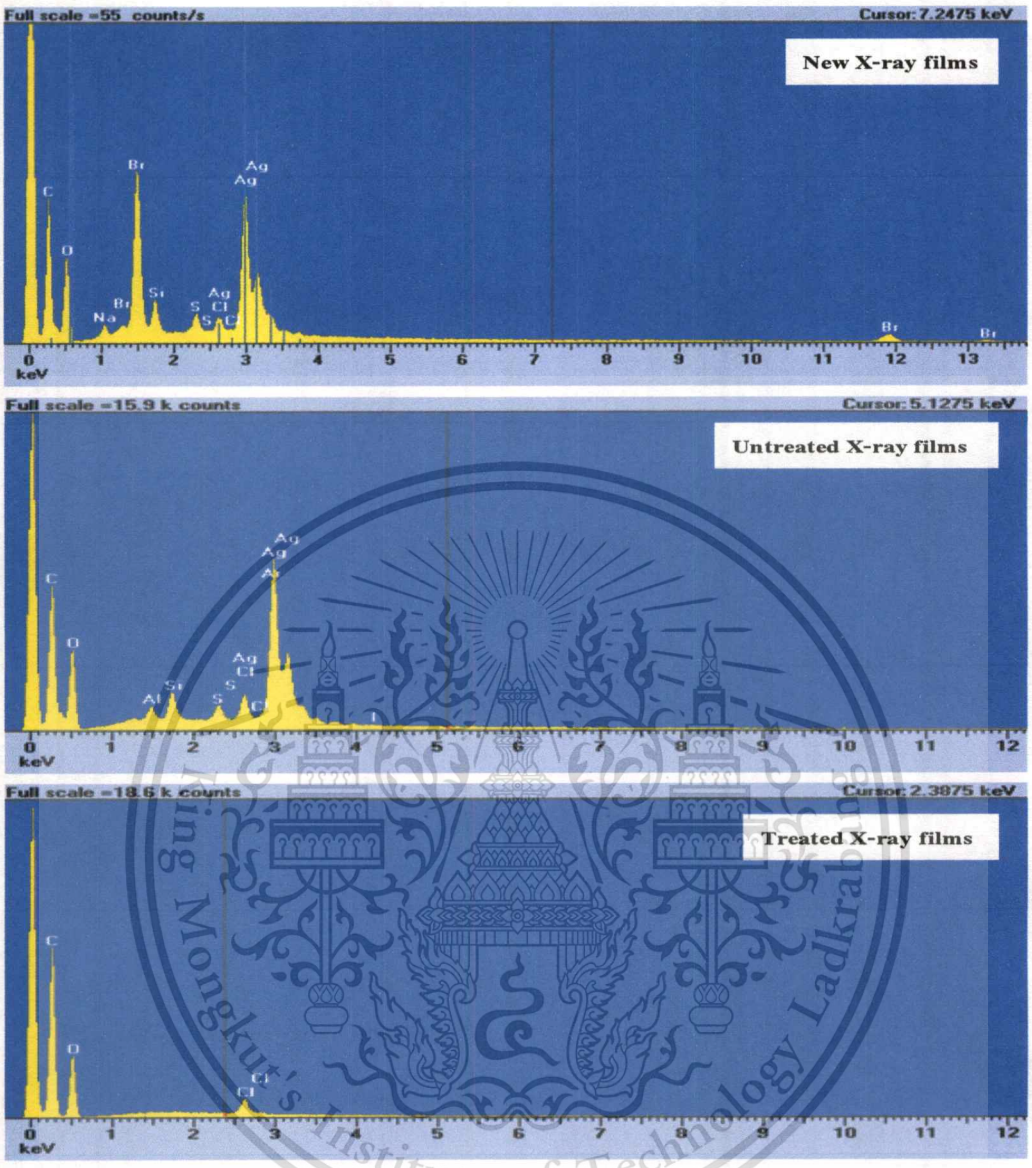

Figure 4.11 Spectrum of X-ray films analyzed by EDS

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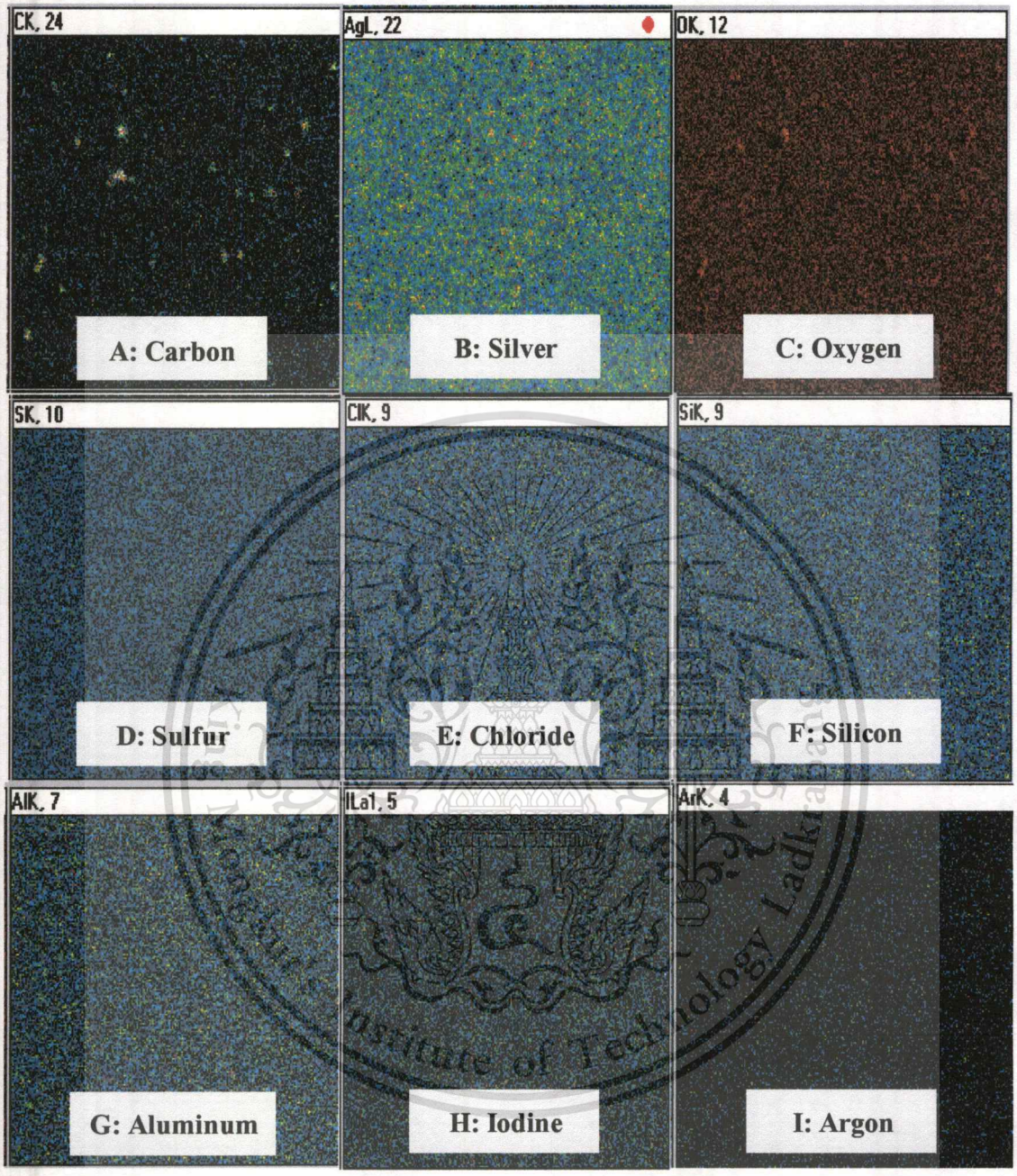

Figure 4.12 Color images of all displayed elements in used X-ray films without enzyme treatment analyzed by SEM-EDS. The nine elements in this image are A: carbon; B: silver; C: oxygen; D: sulfur; E: chloride; F: silicon; G: aluminum; H: Iodine; I: argon.

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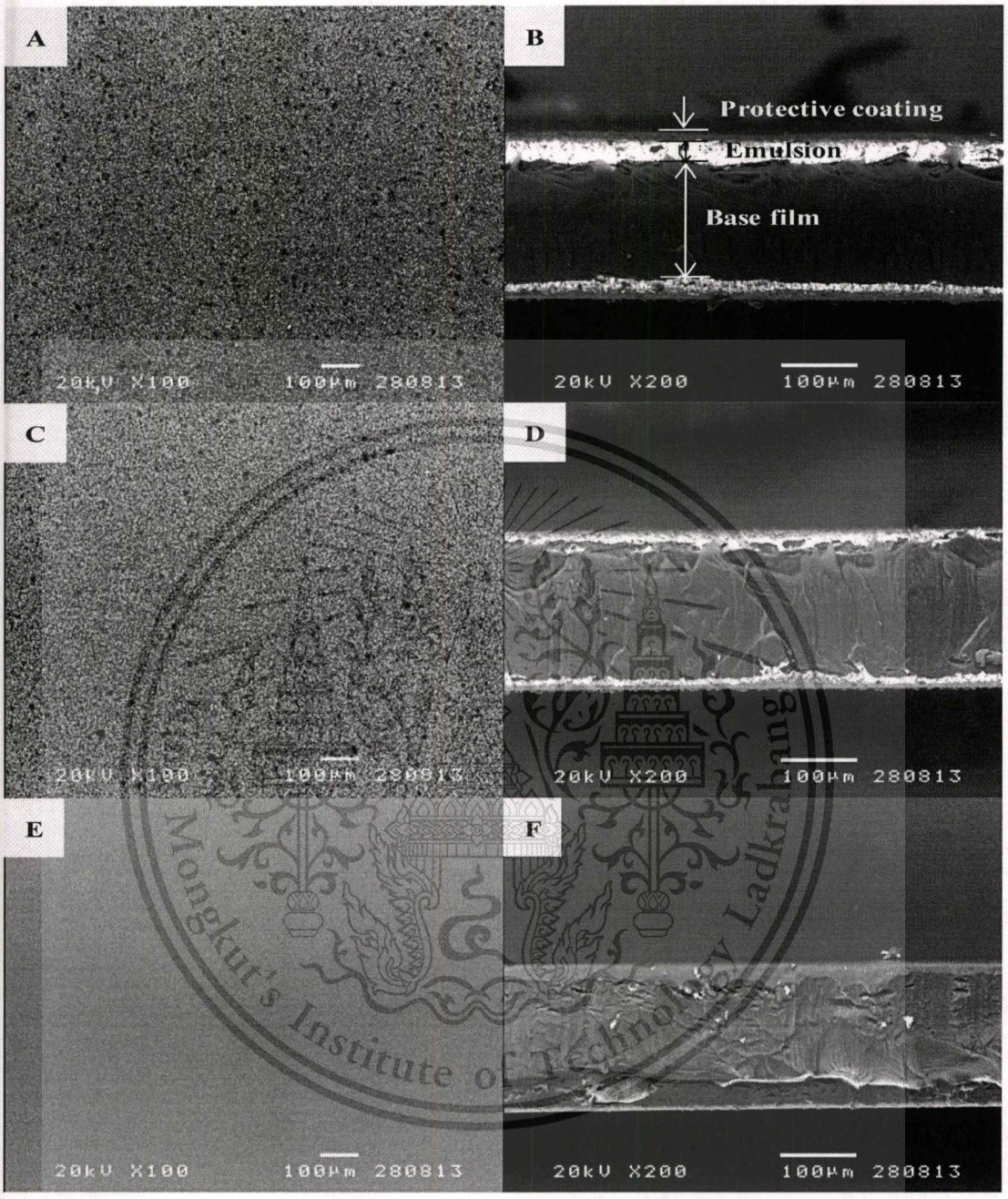

Figure 4.13 SEM micrograph of X-Ray films sheets. A: surface structure of new X-ray films; B: cross-section structure of new X-ray films; C: surface structure of untreated X-ray films; D: cross-section structure of untreated X-ray films; E: surface structure of K-As treated X-ray films; F: cross-section structure of treated X-ray films.

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4.2.4 Reusability of K-As for recovery of silver

Reusability of K-As for silver recovery was evaluated under the optimum conditions at 55°C, pH 4.0, 0.15 U/ml of reaction, no NaCl added in the static condition for 5 min. Based on observation on film clarity (Fig 4.14) and loss weight of film (Table 4.6), the results showed that the enzyme could be reused for at least 25 runs (which maintained 100% of its original activity). The ability of K-As to retain its activity for repeated use makes it suitable for silver recovery. On the other hand, the protease from *Vibrio* sp. (V26) could be reused only for 4 runs and the efficiency was found to drop after the 3rd run while 4th run require more than 40 min (Manjusha, 2011). Shankar *et al.* (2010) reported that the protease from *C. coronatus* was able to be reused for 4 times and also reduced the number of silver recycles. Masui *et al.* (1999) showed that a reuse of proteolytic enzyme in digesting of X-ray films requires the long digesting time. In addition, they found that the greater the concentration of the proteolytic enzyme, the more the reusable cycles of the solution were. However, if K-As was reused more than 25 digesting cycles, activity of the enzyme could be retarded by silver that has been released and accumulated in the solution. With the fact that, K-As can be reused for at least 25 runs at constant hydrolysis time compared with the previously reports, the K-As is capable of withstanding reuse to greater extent for commercial application.

4.2.5 Recovery of silver

The X-ray films treated with K-As resulted in the gelatin layer containing silver bromide grain being stripped off into the solution and clean PET film was achieved. The collected PET film can be reused for production of fabrics, packaging films, soft drink bottles and recording tapes (Radha and Arun, 2010). Weight loss of X-ray film after treatment was observed around 3.1% (w/w) based on initial weight of film. Similar results were shown by some proteases as described previously, with the loss weight between 2-5% (w/w) (Shankar *et al.,* 2010; Manjusha, 2011). The slurry obtained was smelted in furnace at 1000°C for 3 h (Fig 4.15A). Under the obtained conditions, 0.08 g of silver was recovered with 96.8% from 10 g of X-ray films following the procedure as described in the silver recovery method section. Shankar *et al.* (2010) have reported around 0.2% (w/w) of silver recovery from X-ray films. The alkaline protease from *Aspergillus vesrsicolor* was recovered 0.135 g silver from 40 g of used X-ray films. In contrast, although the NaCl activated the activity of K-As, but it

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was found that it was difficult to purify the released silver when NaCl is present in the system (Fig 4.15B). The conditions of pH 4.0, 55°C, 0.15 U/ml of reaction and without NaCl in static condition for 5 min was found to be the most idealistic for the recovery of silver from used X-ray films by K-As.

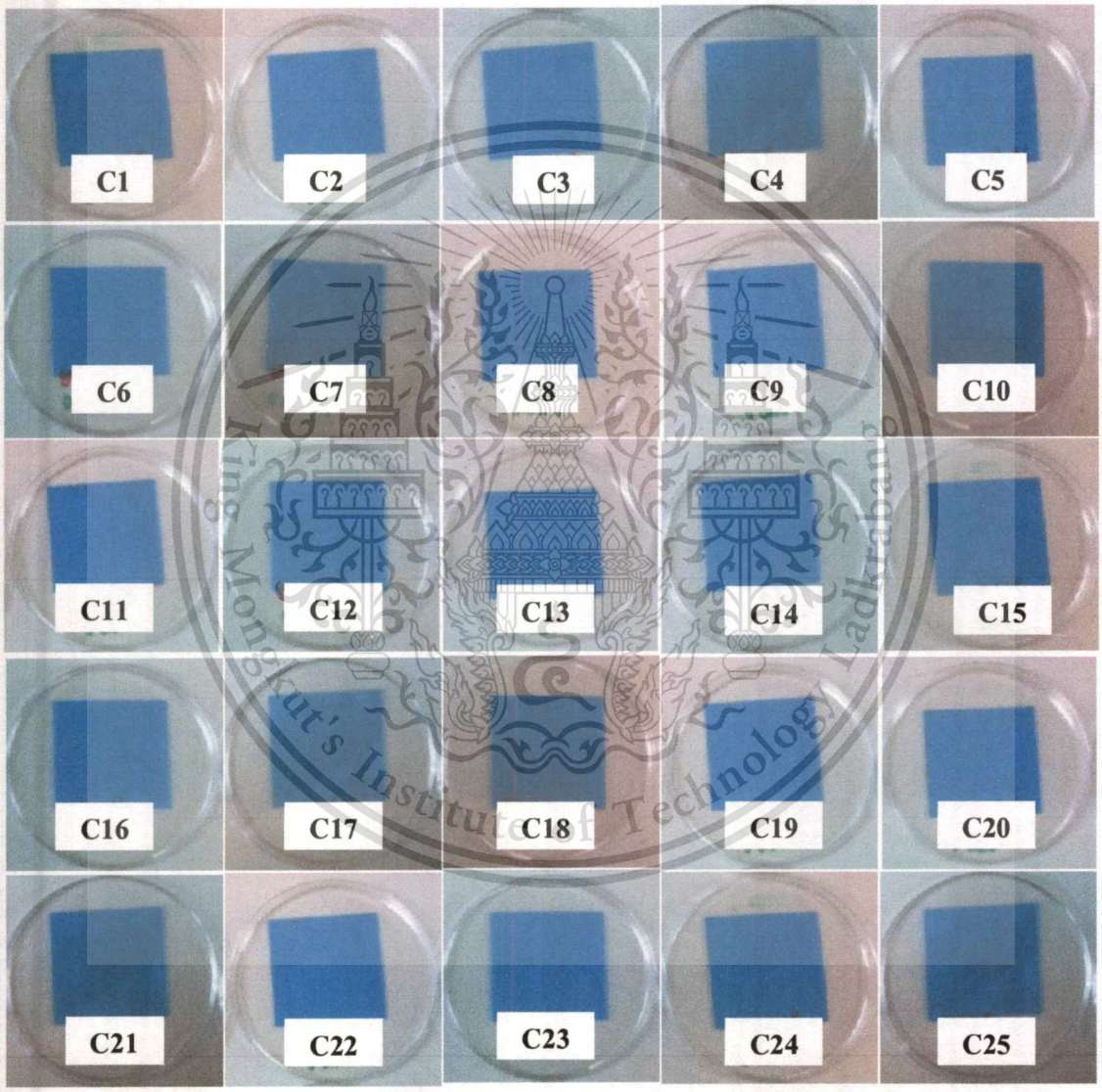

Figure 4.14 Twenty-five sheets of clean bluish polyester obtained after reuse of enzyme for 25 cycle treatment X-ray films by K-As under the optimum conditions (C1-C25).

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was found that it was difficult to purify the released silver when NaCl is present in the system (Fig 4.15B). The conditions of pH 4.0, 55°C, 0.15 U/ml of reaction and without NaCl in static condition for 5 min was found to be the most idealistic for the recovery of silver from used X-ray films by K-As.

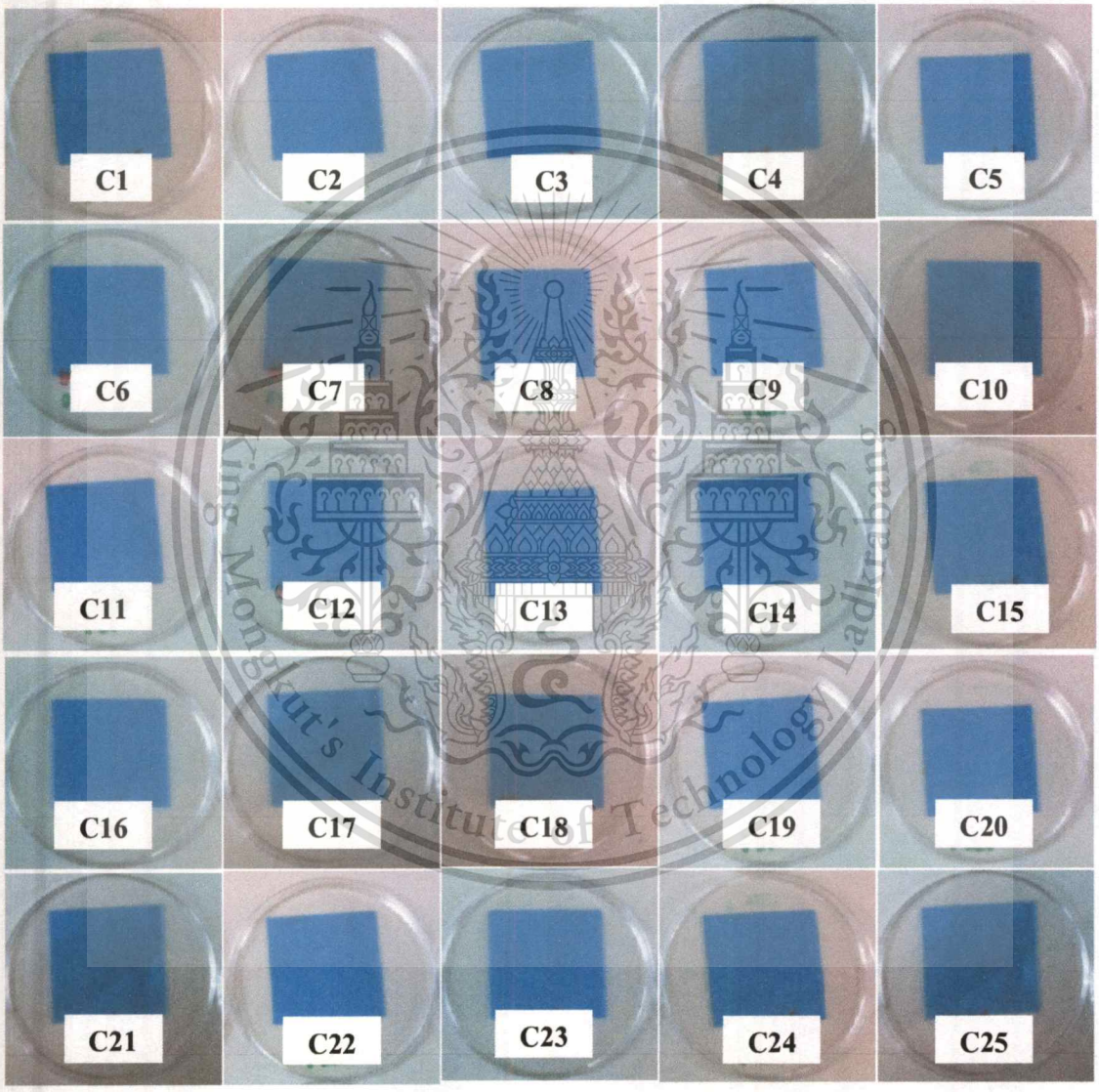

Figure 4.14 Twenty-five sheets of clean bluish polyester obtained after reuse of enzyme for 25 cycle treatment X-ray films by K-As under the optimum conditions (C1-C25).

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Table 4.6 Weight loss and observation of X-ray films after reuse of K-As for 25 cycles under the optimum conditions

Cycle	Wieght loss of X-ray films (g)	Observation
1	0.0038	* Clean PET film obtained
2	0.0036	* Clean PET film obtained
3	0.0038	* Clean PET film obtained
4	0.0033	* Clean PET film obtained
5	0.0031	* Clean PET film obtained
6	0.0031	* Clean PET film obtained
7	0.0034	* Clean PET film obtained
8	0.0033	* Clean PET film obtained
9	0.0036	* Clean PET film obtained
10	0.0036	* Clean PET film obtained
11	0.0033	* Clean PET film obtained
12	0.0031	* Clean PET film obtained
13	0.0036	* Clean PET film obtained
14	0.0033	* Clean PET film obtained
15	0.0033	* Clean PET film obtained
16	0.0034	* Clean PET film obtained
17	0.0033	* Clean PET film obtained
18	0.0036	* Clean PET film obtained
19	0.0031	* Clean PET film obtained
20	0.0033	* Clean PET film obtained
21	0.0033	* Clean PET film obtained
22	0.0036	* Clean PET film obtained
23	0.0034	* Clean PET film obtained
24	0.0031	* Clean PET film obtained
25	0.0031	* Clean PET film obtained

*Clean PET films was obtained in all cycles

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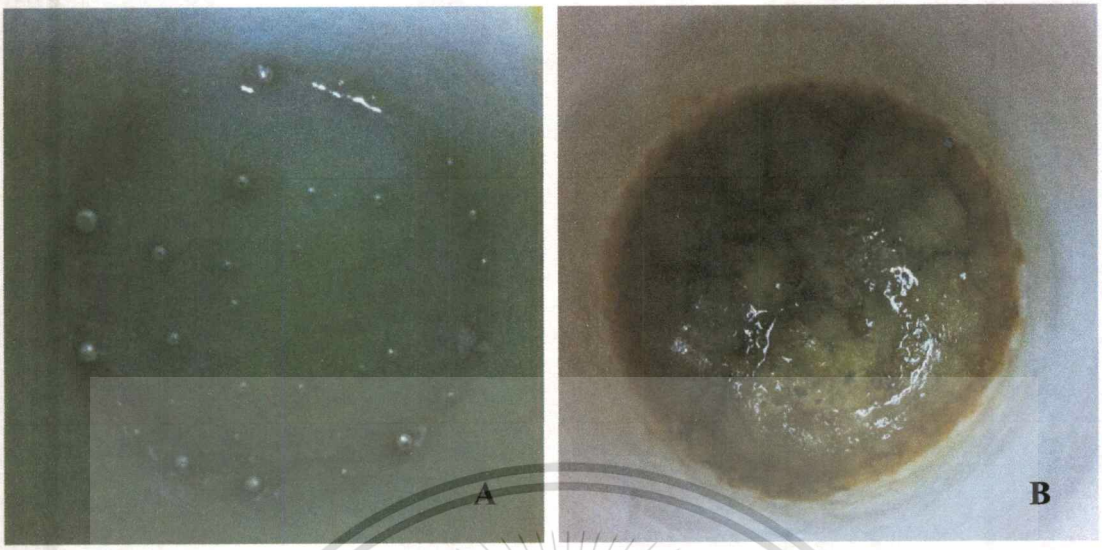

Figure 4.15 Silver grains after smelted at 1000°C for 3 h. A: without NaCl and B: with NaCl in recovery process

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CHAPTER V

CONCLUSIONS

1. Characterization of the Kumamolisin-As was carried out. Kumamolisin-As consisted of two activity bands with molecular masses of 41 and 45 kDa. The optimal temperature, pH and salt concentration for the K-As activity were at 55°C, pH 4.0 and in the presence of 4 M NaCl, respectively. K-As was stable over a wide range of NaCl (1.5 to 4.0 M) at pH 3.0 to 5.0 and 37 to 80°C which maintained more than 80 % of its original activity.

2. A Plackett-Burman's design (PBD) allowed the rapid screening of a large experimental domain for optimization of silver recovery from used X-ray film. A total of four variables include K-As concentration, NaCl concentration, shaking rate and reaction time were screened. The initial screening showed that NaCl concentration, with a *p*-value of 0.0003 was determined to be the most significant factor but it showed negative effect on silver recovery. The other variables were not significant and maintained at constant level, while NaCl was selected for further optimization.

3. Response surface methodology using five-level CCD was used to optimize the silver recovery process. The optimum condition for silver recovery was found to be at pH 4.0, 55°C, 0.15 U/ml of solution, no NaCl added in the static condition for 5 min. Although NaCl increased the activity of K-As, it was found that the silver purification step is difficult when NaCl is present in the system. Under the optimized condition, 0.08 g of silver was recovered from 10 g of X-ray films which was about 97% of silver contained in used X-ray films.

4. K-As could be reused at least 25 runs with no loss of activity was observed. K-As in this study was capable of withstanding repeated use to greater extent than other previously reported enzymes.

5. After exposure to K-As treatment, X-Ray films has undergone obvious changes and reveal extensive smooth surface. Silver crystal and gelatin missing from the X-Ray films surface were evident.

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APPENDICES

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APPENDIX A

ANTIBIOTIC AND IPTG PREPARATION

1. Ampicillin stock solution (1000 mg/ml)

Ampicillin sodium salt	10	g
Sterile distilled water	10	ml

Dissolve 10 g of sodium ampicillin in sterile distilled water to make a final volume of 10 ml. If sterilization is required, prewash a 0.45 or 0.22 μm sterile filter by drawing through 5-10 ml of sterile dH_2O. Then pass the ampicillin solution through the washed filter. Store the ampicillin in aliquots at -20°C for 1 year (or at 4°C for 3 months).

2. IPTG stock solution (1 Molar)

IPTG	2.38	g
Sterile distilled water	10	ml

Dissolve 2.38 g of IPTG in 8 ml of distilled H_2O. Bring to a final volume of 10 ml with sterile dH_2O. Filter sterilize with a 0.45- or 0.22 μm sterile syringe filter. Store the IPTG in 1mL of aliquots at -20 °C.

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APPENDIX B

CULTURE MEDIA AND PREPARATION METHODS

1. Luria-Bertani broth (LB-B)

LB Agar, Miller and LB Broth, Miller are based on the formula described by Miller (1972). This medium is used for the growth and maintenance of *Escherichia coli* strains used in molecular microbiology procedures. LB Broth, Miller is a nutritionally rich medium designed for growth of pure cultures of recombinant strains. *E. coli* is grown more rapidly to late log phase in LB Medium because they provide the cells with amino acids, nucleotide precursors, vitamin and others metabolites. Some plasmid vectors replicate to high copy numbers without selective amplification. Some vectors do not replicate so freely, and need to be selectively amplified.

Ingredients in 1 Litre

Tryptone	10	g.
Yeast extract	5	g.
NaCl	10	g.

Dissolve and adjust pH to 7.0 with NaOH and HCl and distribute into tubes or screw-capped (6 mm) bottles to a depth of about 16 mm (5 ml). Sterile by autoclaving at 121 °C, 15 pounds/inch^2, and pressure for 15 min.

For the LB broth supplement with 50 μg/ml of ampicillin, the medium was cool down to room temperature. Then, add antibiotics, mix in a laminar air flow cabinet before use.

2. Luria-Bertani Agar (LB-A)

Ingredients in 1 Litre

Tryptone	10	g.
Yeast extract	5	g.
NaCl	10	g.
Agar	16	g.

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Dissolve and adjust pH to to 7.0 with NaOH and HCl. Sterile by autoclaving at 121 °C, 15 pounds/inch^2, and pressure for 15 min.

For the LB medium supplemented with antibiotic (50 μg/ml of ampicillin). Cool down the medium to 50-60 °C. Then, add antibiotics, mixed and pour plates in a laminar air flow cabinet. Store at 4 °C in the dark and do not store longer than 3 months. If the medium was store in 4 degree for long time, please don't add antibiotics. The antibiotics could be added ahead of time. Make it fresh always. More than one month store the antibiotics is not good.

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APPENDIX C

STANDARD ASSAY METHOD

1. Determination of Protein

The protein concentration was measured by the method of Lowry *et al.* (1951). Bovine serum albumin was used as a standard. The most accurate method of determining protein concentration is probably acid hydrolysis followed by amino acid analysis. Most other methods are sensitive to the amino composition of the protein, and absolute concentrations cannot be obtained. The procedure of Lowry *et al* is no exception, but its sensitivity is moderately constant from the protein to the protein, and it has been so widely used that Lowry protein estimations are a completely acceptable alternative to a rigorous absolute determination in almost all circumstances in which protein mixtures or crude extracts are involved.

The method is based on the Biuret reaction, in which the peptides bonds of protein react with copper under alkaline conditions to produce Cu^{+}, which react with the Folin reagent, and the Folin-Ciocalteau reaction, which is poorly understood but in essence phosphomolybdotungstate is reduced to heteropolybdenum blue by the copper-catalyzed oxidation of aromatic amino acids. The reactions result in a strong blue color, which depends partly on the tyrosine and tryptophan content. The method is sensitive down to about 0.01 mg of protein/ml, and its best used on solutions with concentrations in range 0.01 to 1.0 mg/ml of protein.

1.1 Reagents

Solution A: 2% (w/v) Na_2CO_3 in 0.1 N NaOH (room temp. for 4 month)

Solution B: 0.5 % (w/v) $CuSO_4 \cdot 5\ H_2O$ in 1% (w/v) sodium citrate (4 °C in the dark)

Solution C: Folin phenol reagent : distilled 1:1 (v/v) (make fresh)

Solution D: 50 ml of solution A : 1 ml of solution B 50:1 (v/v) (make fresh)

Standard Bovine Serum Albumin (BSA)

Bovine serum albumin	10	mg
Distilled water	10	ml

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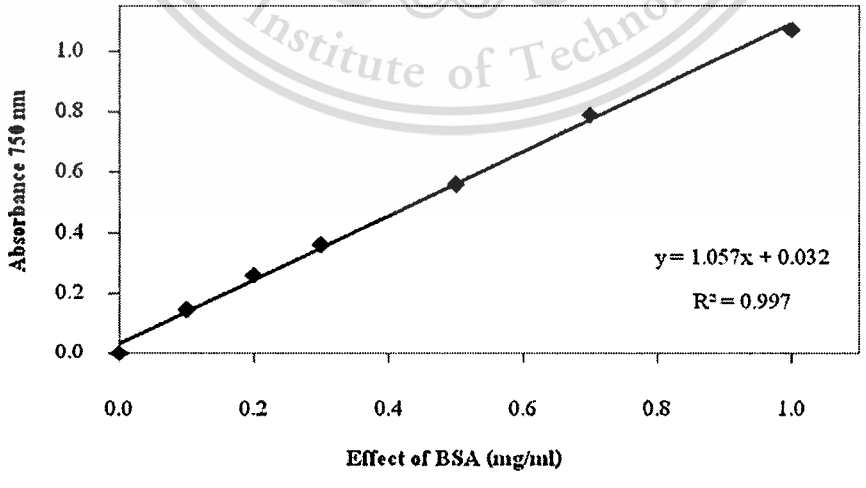
Dissolve and adjust to the volume with distilled water. The solution can be stored at -20°C for 4 month.

1.2 Procedure

Different dilutions of BSA solutions are prepared by mixing stock BSA solution (1 mg/ ml) and water in the test tube. The BSA range is 0 to 1 mg/ ml. This is used for standard curve.

A 100 µl of sample containing protein between 0.2 to 0.7 mg/ml was prepared. If the approximate sample concentration is unknown, a range of dilutions (1, 1:1, 1:5, 1:10, 1:100) should be assayed. Each sample was prepared in duplicates. For the calibration curve, the duplicated volumes of 10, 20, 30, 50, 70, 100 µl of 1 mg/ml BSA standard solution into test tube, and make each up to 100 µl with distilled water were prepared. A 100 µl of distilled water into an additional tube was provided as a reagent blank.

A 1 ml of solution D was added to each tube, and mixed the solution well by gentle vortexing. This solution is incubated at room temperature for 10 min. A 100 µl of solution C was again added to each tube, and then mixed well and incubated for 30 min at room temperature. Zero the colorimeter with blank and take the optical density (measure the absorbance) at 750 nm between 0 to 15 min after incubated for 30 min. Plot the absorbance against protein concentration to get a standard calibration curve. Check the absorbance of sample and determined the concentration of the sample using the standard curve plotted.



Standard curve of bovine serum albumin (BSA)

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2. Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE is the most widely used method for qualitatively analyzing protein mixtures. It is particularly useful for monitoring protein purification, and because the method is base on the separation of proteins according to size, the method can also be used to determine the relative molecular mass of proteins.

Samples to be run on SDA-PAGE are first boiled for 5 min in sample buffer containing β-mercaptoethanol and SDS. The mercaptoethanol reduces any disulfide bridges present that are holding together the protein tertiary structure. SDS (CH_3-$[CH_2]_{10}$-$CH_2OSO_3Na^+$) is an anionic detergent and binds strongly to, and denatures, the protein. Each protein in the mixture is therefore fully denatured by this treatment and opens up into a rod-shaped structure with a series of natively charged SDS molecular along the polypeptide chain. On average, one SDS molecular is therefore completely swamped by the SDS molecules. The sample buffer also contains an ionizable tracking dye usually bromophenol blue that allows the electrophoresis buffer to the bottom when injected into the loading well. When the main separating gel has been poured between the glass plates and allowed so set, a shorter stacking gel is poured on top of the separating gel, and it is into this gel that the wells are formed and the proteins loaded. Once all samples are loaded, a current is passed through the gel. Once the protein samples has passed through the stacking gel and have entered the separating gel, the negatively charged protein-SDS complexes continue to move toward the anode and because they have the same charge per unit length they travel into the separating gel under the applied electric field with the same mobility. However, as they pass through the separating gel the proteins separate, owing to the molecular sieving properties of the gel. Quite simply, the smaller the protein, the more easily it can pass through the separating gel the pores of the gel, whereas large proteins are successively retarded by frictional resistance owing to the sieving effect of the gel. Being a small molecule, the bromophenol blue dye is totally unretarded and therefore indicates the electrophoresis front. When the dye reaches the bottom of the gel the current is turned off and the gel is removed from between the glass plates, shaken in an appropriate stain solution (usually Coomassie brilliant blue) for a few hours, and then washed in destain solution overnight. The destain solution removes unbound background dye from gel, leaving stained proteins visible as blue bands on a clear background.

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2.1 Reagent

Monomer solution

Acrylamide	30 %	(w/v)
Bisacrylamide	0.8 %	(w/v)

Dissolved and made up to 100 ml with deionized water

Note: Acrylamide is a potential neurotoxin and should be treated with great care. Its effects are cumulative, and therefore, regular users are at greatest risk. In particular, take care when weighing out acrylamide. Do this in a fume hood, and wear an appropriate face mask. The solution can be stored up to 3 months at 4 °C in the dark.

4x Resolving gel buffer

Tris (hydroxymethyl) aminomethane	18.15	g
Deionized water	90	ml

Dissolved and adjusted the pH to 8.8 by using 0.1 N HCl. Made up to 100 ml with deionized water.

Note: The solution can be store up to 3 months at 4 °C in the dark.

4x Stacking gel buffer

Tris (hydroxymethyl) aminomethane	6	g
Deionized water	90	ml

Dissolved and adjusted the pH to 6.8 by using 0.1 N HCl. Made up to 100 ml with deionized water.

Note: The solution can be store up to 3 months at 4 °C in the dark.

10% (w/v) Sodium dodecyl sulphate (SDS)

Sodium dodecyl sulphate	10	g
Deionized water	90	ml

Dissolved and made up to 100 ml with deionized water.

Note: SDS come out of solution at low temperature, and this can even occur in a relatively cold laboratory. If this happens, simply warm up the bottle in a water bath. The solution can be store up to 6 months at room temperature.

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10x Tank buffer for SDS-PAGE

Tris (hydroxymethyl) aminomethane	30.28	g
Glycine	144.13	g
Sodium dodecyl sulfate	10	g
Distilled water	90	ml

Dissolved and made up to 1 litter with distilled water.

Note: Diluted 10 times before use. The solution can be store up to 1 month at room temperature.

2x Sample buffer for SDS-PAGE

4x Stacking gel buffer	2.5	ml
Glycerol	2	ml
Bromophenol blue (2 mg/ml)	4	ml
β-mercaptoethanol	0.2	ml

Dissolved and made up to 10 ml with deionized water.

Note: The reagent should be filtered through Whatman No. 1 filter paper before use.

10% (w/v) Ammonium persulfate

Ammonium persulfate	20	mg
Deionized water	180	µl

Note: Make fresh the reagent before use

Staining solution

Coomassie brilliant blue (R-250)	1.25	g
Ethanol	450	ml
Acetic acid	100	ml

Dissolved and made up to 1 litter with distilled water.

Note: The reagent should be filtered through Whatman No. 1 filter paper before use. The solution can be store in the dark at room temperature.

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Destaining solution

Methanol	300	ml
Acetic acid	100	ml

Dissolved and made up to 1 litter with distilled water.

12.5% Running gel for SDS-PAGE (for 2 gel)

Deionized water	4.1314	ml
4x Running gel buffer	3.25	ml
Monomer solution	5.4171	ml
10% (w/v) SDS	130	µl
10% (w/v) Ammonium persulfate	65	µl
TEMED	6.5	µl

4% Stacking gel for SDS-PAGE (for 2 gel)

Deionized water	3.053	ml
4x Stacking gel buffer	1.25	ml
Monomer solution	667	µl
10% (w/v) SDS	50	µl
10% (w/v) Ammonium persulfate	25	µl
TEMED	6.5	µl

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