การเพิ่มการผลิตอะไมเลสของ *Xanthomonas campestris* TISTR 840 และผลต่อการผลิตแซนแทนกัม

นางสาวปรมาภรณ์ เกิดทรัพย์

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IMPROVING AMYLASE PRODUCTION OF *Xanthomonas campestris* TISTR 840 AND ITS EFFECTS ON XANTHAN GUM PRODUCTION

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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ปรมาภรณ์ เกิดทรัพย์ : การเพิ่มการผลิตอะไมเลสของ *Xanthomonas campestris* TISTR 840 และผลต่อการผลิตแซนแทนกัม (IMPROVING AMYLASE PRODUCTION OF *Xanthomonas campestris* TISTR 840 AND ITS EFFECTS ON XANTHAN GUM PRODUCTION) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร.สุเมธ ตันตระเธียร, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ.ดร.รมณี สงวนดีกุล, อ.ดร.จันทร์ประภา อิ่มจงใจรัก, 132 หน้า.

แป้งมันสำปะหลังดิบถูกนำมาใช้เป็นแหล่งคาร์บอนในการเลี้ยงเชื้อ *Xanthomonas campestris* เพื่อผลิตแซนแทนกัมเนื่องจากมีราคาถูกและมีการผลิตเป็นจำนวนมากในประเทศไทย จากการทดลองเลี้ยงเชื้อในถังหมักขนาด 5 ลิตร ควบคุม pH เท่ากับ 7 อุณหภูมิ 30 องศาเซลเซียส อัตราการให้อากาศ 0.5 ลิตรต่อลิตรต่อนาที มีการกวนที่ 200 รอบต่อนาที เป็นเวลา 120 ชั่วโมง พบว่าเชื้อสามารถเจริญได้ในอาหารชนิด XOL ที่ใช้แป้งมันสำปะหลัง 1 เปอร์เซ็นต์ ที่ผ่านการทำให้สุกด้วยความร้อนเป็นแหล่งคาร์บอน เนื่องจากเชื้อสามารถผลิตเอนไซม์อะไมเลสออกมาย่อยแป้งได้ ซึ่งเชื้อผลิตอะไมเลสได้สูงสุด 0.350 หน่วยต่อมิลลิลิตร ในชั่วโมงที่ 16 ของการเลี้ยง และผลิตแซนแทนกัมได้ 9.06 กรัมต่อลิตร แต่การใช้แป้งสุกดังกล่าวทำให้ในขั้นตอนการตกตะกอนแซนแทนกัมมีสารโมเลกุลเล็กปนเปื้อนมากถึงร้อยละ 48.97 จึงทดลองใช้แป้งมันสำปะหลังดิบ 1 เปอร์เซ็นต์ แทนการใช้แป้งสุก พบว่าเชื้อมีการเจริญที่ช้าและผลิตอะไมเลสได้ต่ำกว่าการใช้แป้งสุกคือผลิตได้ 0.277 หน่วยต่อมิลลิลิตร และผลิตแซนแทนกัมได้ต่ำกว่าการใช้แป้งสุกอยู่ 8 เท่า (2.23 กรัมต่อลิตร) แต่แซนแทนกัมที่ผลิตได้มีสารโมเลกุลเล็กปนเปื้อนเพียงร้อยละ 25.95 นอกจากนี้ยังพบว่าเมื่อเลี้ยงเชื้อ *X. campestris* ด้วยแป้งมันสำปะหลังดิบ การเติมเปปโตนลงในอาหารเลี้ยงเชื้อมีผลต่อการเจริญอย่างมีนัยสำคัญ การปรับปริมาณแป้งดิบโดยปรับอัตราส่วนคาร์บอนต่อไนโตรเจนในระบบและการปรับอัตราการให้อากาศจึงถูกนำมาใช้เพื่อเพิ่มความสามารถในการผลิตอะไมเลสของ *X. campestris* โดยปรับอัตราส่วนคาร์บอนต่อไนโตรเจนเป็น 10:1 20:1 และ 30:1 และอัตราการให้อากาศเป็น 0.0 0.5 และ 1.0 ลิตรต่อลิตรต่อนาที จากการทดลองโดยวางแผนการทดลองแบบ Central Composite Design พบว่าการเพิ่มอัตราส่วนคาร์บอนต่อไนโตรเจนร่วมกับการเพิ่มอัตราการให้อากาศส่งผลให้เชื้อผลิตอะไมเลสได้สูงขึ้น ซึ่งทำให้ปริมาณแซนแทนกัมสูงขึ้นตามไปด้วย และสภาวะที่เหมาะสมที่สุดที่ทำให้เชื้อผลิตอะไมเลสและแซนแทนกัมได้สูงสุดอยู่ที่จุดเดียวกันคือที่อัตราส่วนคาร์บอนต่อไนโตรเจน 30:1 และให้อากาศที่ 1.0 ลิตรต่อลิตรต่อนาที โดยผลิตอะไมเลสและแซนแทนกัมได้ 0.344 หน่วยต่อมิลลิลิตร และ 7.947 กรัมต่อลิตร ตามลำดับ เนื่องจากการทำให้เชื้อผลิตอะไมเลสได้มากขึ้นส่งผลให้ได้ปริมาณแซนแทนกัมสูงขึ้น ในขั้นสุดท้ายจึงทำการปรับปรุงสายพันธุ์ *X. campestris* โดยการกลายพันธุ์ด้วยเอทิลมีเทนซัลโฟเนต และคัดเลือกสายพันธุ์กลายที่ผลิตอะไมเลสได้มากกว่าปกติมาทดลองเทียบกับสายพันธุ์ปกติพบว่า เชื้อมีการเจริญและการผลิตแซนแทนกัมสูงกว่าสายพันธุ์ปกติอย่างมีนัยสำคัญคือผลิตได้ 5.97 กรัมต่อลิตร ในขณะที่สายพันธุ์ปกติผลิตได้ 4.31 กรัมต่อลิตร

สาขาวิชา.....เทคโนโลยีชีวภาพ..... ลายมือชื่อนิสิต.....ปรมาภรณ์ เกิดทรัพย์.....

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PARAMAPORN KERDSUP : IMPROVING AMYLASE PRODUCTION OF *Xanthomonas campestris* TISTR 840 AND ITS EFFECT ON XANTHAN GUM PRODUCTION. ADVISOR : ASSOC. PROF. SUMATE TANTRATIAN, Ph.D., CO-ADVISOR : ASST. PROF. ROMANEE SANGUANDEEKUL, Ph.D., CHANPRAPA IMJONGJIRAK, Ph.D., 132 pp.

Raw cassava starch was used as a carbon source to produce xanthan gum by *Xanthomonas campestris* because its availability in Thailand. The first experiment was done in 5 liter bioreactor with 3 liter XOL medium containing 1% gelatinized cassava starch that was heat till the solution temperature reach to 80°C. The incubation condition was controlled at pH = 7.0, 30°C, aeration rate 0.5 vvm, and stirred at 200 rpm. It was found that the bacteria could hydrolyze starch by secreted amylase. The highest amylase activity, 0.350 U/ml was found at 16 hour of cultivation and xanthan gum yield was 9.06 g/l. However, using gelatinized starch gave high impurity, the small molecule substance, in crude xanthan gum. There was 48.97% of impurity was found in the crude xanthan gum. From this reason, 1% of raw cassava starch was used instead of gelatinized form. The result showed amylase activity (0.277 U/ml) was lower than cultured in gelatinized starch medium. Moreover, using raw starch gave 4 folds lower of xanthan gum yield (2.23 g/l) than using gelatinized form. However, the impurity found in crude xanthan gum produced from raw starch was 25.95%, it was significantly lower than culturing in gelatinized starch. Moreover, production of xanthan gum by raw cassava starch needed peptone as a growth factor. The variation of raw starch content by adjusted C:N ratio in the system and aeration rate were used to improved ability to produced amylase of *X. campestris*. The C:N ratio was adjusted as 10:1, 20:1, and 30:1, and aeration rate at 0.0, 0.5. and 1.0 vvm were used in the Central Composite Design experiment. The result showed that increasing of C:N ratio together with aeration rate increased amylase production of the bacteria and the xanthan gum was also increased. The optimum condition for highest amylase and xanthan gum production was in the same point at C:N = 30 and applied aeration rate at 1.0 vvm. In this condition, the *X. campestris* produced highest amylase activity and xanthan gum at 0.344 U/ml and 7.947 g/l, respectively.

Mutation of *X. campestris* with ethyl methansulfonate (EMS) was also used to improve new strain that could overproduce amylase. The mutant strain was selected by starch agar plate and cultured in 5 liter bioreactor to compare the ability of amylase and xanthan gum production with wild-type strain. It was found that the mutant could produce xanthan gum with significantly higher than the wild-type. The mutant and wild-type produced 5.97 and 4.31 g/l of xanthan gum, respectively.

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CHAPTER I

INTRODUCTION

Xanthan gum is an industrial important hetero-polysaccharide produced by *Xanthomonas campestris*. It has high viscosity at low concentration and stability over wide ranges of temperature, pH, and ionic strength. Xanthan gum has application in food, pharmaceutical, paint, and pesticide industries and also in oil recovery (Sutherland, 1990).

Since 1935, *X. campestris* was known as bacterium which was parasite on plants (Duc *et al*., 1978). The studies on the molecular biology of *X. campestris*, Tseng and Peng (1985) tried to establish a self-cloning system in this organism. Therefore, genes of certain enzymes were detected by simple procedures. The researchers found that this bacterium possessed starch-hydrolyzing activity and produced amylase as extracellular enzyme. The amylase production by *X. campestris* was turned to be inducible when grown in maltose and starch (Tseng and Peng, 1985)

This showed that *X. campestris* has ability to utilize polysaccharide from many sources to produce xanthan gum. Lilly, Wilson, and Leach (1958) reported that *X. campestris* could grown and produced polysaccharide in medium contained sugar and starch, such as glucose, sucrose and soluble starch from corn. Beside that sorghum flour (Weber and Horam, 1966), dry-milled cornstarch (Miescher, 1969), and corn flour (Duc *et al.*, 1978) were used as carbon source for xanthan gum production but glucose and sucrose are still the best substrate. However, there are

many researcher tried to produce xanthan gum by less expensive substrate to reduce production cost. Several reports showed the effect of agricultural waste and wastewater as substrate for xanthan gum production (Yoo and Harcum, 1999; Lopez, Moreno, and Ramos-Cormenzana, 2001; Lopez *et al*., 2004). They indicated that type and the amount of carbon in the media were the important factors for yield and properties of xanthan gum.

Cassava is an important crop in tropical regions, it ranks forth after rice, wheat and corn as source of complex carbohydrate (Moorthy and Mathew, 1998; Beleia, Butarelo, and Silva, 2006). In Thailand, cassava is the third most important crop because it has excellent drought tolerance properties and can be planted in almost all types of soil. The total production of cassava root in Thailand in 2006 was greater than 35 million tones. The availability and low-priced of cassava starch produced in Thailand make this starch as interested carbon source for xanthan gum production.

Some factors necessary for xanthan gum production were reported by many researcher (De Vuyst, Van, and Vandamme, 1987; Funahashi *et al*.,1987; Zhao *et al*., 1991). They suggested that higher xanthan gum concentration could be obtained by controlling the C:N ratio in both growth and production phases. It was considered that a low C:N ratio was required in the growth phase to achieve both high cell concentrations which in turn could result in higher xanthan gum production rates. The C:N ratio also affected on amylase production in bacteria and mold. The second interesting factor was aeration rate, it is important for growth and xanthan gum production by *X. campestris*. In general, the dissolved oxygen concentration in fermentation system is an important factor on bacterial growth, amylase production

and xanthan gum production in case of *X. campestris*. To apply excess oxygen for stirred bioreactor, the rate of oxygen transfer is influenced by the air flow rate, or aeration rate, and stirrer speed, or agitation speed.

Although, the alternation of parameters for amylase and xanthan gum production is necessary to produce amylase and xanthan gum production by *X. campestris*, induce mutation of the culture can also be concerned. Chemical mutation is the random mutation that usually used to improve some properties of bacteria. Ethyl methanesulfonate (EMS) is a chemical agent that usually use for bacterial mutation.

In this research, we studied the effect of gelatinized and raw cassava starch on amylase and xanthan gum production in stirred bioreactor. Effect of C:N ratio and aeration rate on amylase activity during time and xanthan gum yield were observed. The relationship between amylase and xanthan gum production was also observed. The mutation of *X. campestris* with EMS for cells with ability to overproduce amylase was performed. The ability to produce amylase, growth and xanthan gum production was observed.

CHAPTER II

LITERATURE REVIEW

2.1 **Cassava and cassava starch**

2.1.1 History and background

The cassava plant has been classified as *Manihot utilissima* Pohl of the family Euphorbiaceae. Somehow, the name *Manihot esculenta* is being increasingly adopted. Cassava is the term usually applied in Europe and in the United States to the roots of the plant, whereas, tapioca is the name given the processed products of cassava. The cassava plant was first believed to have been cultivated in the tropical regions of North and South America. The roots from the plant are an important carbohydrate food source in many regions of the tropics (Corbishley and Miller 1984).

2.1.2 Cassava production in Thailand

Cassava (*Manihot esculenta Crantz*) is the third most important crop in Thailand. The root crop is known by many name in Thailand, but cassava and tapioca are the most widely used. Cassava was introduced into the southern part of

Thailand from Malaysia during 1786 – 1840 and was gradually distributed throughout the country within a few years. The main concentration of the crop is now found in the northeast of Thailand, especially in Nakhon Ratchasima province. Cassava has excellent drought tolerance properties and can be planted in almost all types of soil. Therefore, the planted area has rapidly increased. Cassava is grown by a large number of farmers, who own small plots of land (about 0.5-2 hectare). No organized large-scale plantations have been established in Thailand, as this is prohibited by the land reform act. The total acreage of cassava, which peaked at about 1.6 million ha in 1988 to 1989 is now reduced to 1.05 million ha (Table 2.1). This trend is driven by a national agricultural policy promoting an increase in yields with unchanged planted area (Sriroth, Lamchaiyaphum, and Piyachomkwan, 2007).

Table 2.1 Planted area, production and yield of cassava in Thailand during 1994/1995 to 2004/2005.

Year	Planted area (ha)	Total production (tonnes)	Yield (t/ha)
1994/95	1,245,157	16,217,378	13.02
1995/96	1,228,114	17,387,780	14.16
1996/97	1,230,381	18,083,579	14.70
1997/98	1,119,096	15,968,474	14.27
1998/99	1,172,374	16,057,000	14.77
1999/00	1,095,631	19,064,000	15.45
2000/01	1,106,880	18,396,000	17.53
2001/02	999,040	16,868,000	17.66
2002/03	1,029,600	19,718,000	19.29
2003/04	1,081,120	21,440,000	20.27
2004/05	1,043,840	16,938,000	17.18

Source: Office of Agricultural Economics (2005)

2.1.3 Cassava Starch and Starch-based Industries

At the time that cassava was introduced into the southern part of Thailand (1786 - 1840), a cottage-scale industry for production of cassava meal or cassava flour was adopted from neighboring countries, Malaysia and Singapore. Conversion of fresh cassava roots, by grating, mixing with water followed by sedimentation and sun-drying (or conductive heating) produces a product traditionally called “cassava flour” but now called “cassava starch”. Cassava starch may be further processed to make sago pearl, which is a traditional dessert for the people in the southern part of Thailand. (Sriroth, Lamchaiyaphan, and Piyachomkwan 2007)

Demand for cassava starch increased dramatically and this led to the development of the modern starch manufacturing process in the 1970s. A survey conducted in 1996 indicated that at that time there were 41 modern factories registered to the Thai Tapioca Flour Industries Trade Association. These factories were working with modern separation and drying processes. The processing time (from the grating of fresh root to dried starch) is estimated to be less than 30 minutes. Presently, factories using the sedimentation process do not exist in Thailand.

About 40% of cassava starch, i.e. 600,000-800,000 tons, is used domestically (Table 2.2) and 60%, i.e. 700,000-900,000 tons, for export.

Table 2.2 Annual demand of cassava starch for the production of sweeteners and MSG/lysine in Thailand.

Products	Quantity of starch used (tonnes/year)	Product (kg/kg of starch)
High fructose	54,000	1.00
Glucose syrup	60,000	0.90-0.95
Dextrose monohydrate	20,000	1.75
Dextrose anhydrous	500	0.50
Sorbitol	30,000	1.20
MSG/Lysine	232,980	0.42

Source: Sriroth (1998).

2.1.4 Starch composition

Since 1984, and in parallel with the growth of the cassava industry in Thailand, several new cultivars suitable for industrial purpose have been developed, including Rayong 3, Rayong 60, Rayong 90 and Kasetsart 50. The proximate composition of cassava starches were presented in Table 2.3.

Table 2.3 Proximate analysis of cassava starches.

Cultivars	Protein (%w/w)	Lipid (%w/w)	Ash (%w/w)	Phosphorus (mg/kg)
Rayong 1	0.17 ± 0.04	nil	0.10 ± 0.02	2.45 ± 0.08
Rayong 60	0.15 ± 0.02	0.01	0.15 ± 0.04	2.20 ± 0.14
Rayong 90	0.28 ± 0.06	nil	0.08 ± 0.01	2.04 ± 0.05
KU 50	0.30 ± 0.04	0.01	0.15 ± 0.02	2.04 ± 0.05

Source: Sriroth *et al*. (1999)

Normal native starches consist of mixture of 15 – 30% amylose and 70 – 80% amylopectin. Amylose structurally is a linear polymer of anhydroglucose units, of molecular weight approximately between 40,000 and 340,000, the chains containing 250 to 2000 anhydroglucose units. Amylopectin is considered to be composed of anhydroglucose chains with many branch points. The molecular weight may reach as high as 80,000,000. Amylose contributes to the gelling property of starch whereas amylopectin contributes high viscosity (Whistler, BeMiller, and Paschall, 1984). Amylose content in several types of starch showed in Table 2.4.

Table 2.4 Amylose content in several types of starch

Starch Source	% Amylose
Waxy Rice	0
High Amylose Corn	70
Corn	28
Cassava	17
Waxy Sorghum	0
Wheat	26
Sweet Potato	18
Arrowroot	21
Sago	26
Potato	20

Source: International starch trading (2003)

2.2 Starch hydrolyzing enzyme

2.2.1 Brief overview

Starch is one kind of carbohydrate. Most of starches have amylose and amylopectin in their structure. The monomeric building blocks of amylose and amylopectin and their polymerization to oligomers and polymers has been mentioned. The linkage between monomeric sugar, such as glucose, to disaccharides, is termed a glycosidic linkage. Carbohydrases hydrolyze the specific glycosidic linkage of certain monosaccharide residues. They are able to cleave short-chain oligosaccharides as well as polysaccharides with various structures (Manner, 1979). Table 2.5 shows some of the products of enzymatic hydrolysis of some oligosaccharides. Table 2.6 shows some naturally occurring polysaccharides, their monomeric building blocks, their linkages, and the enzymes used for their scission.

Table 2.5 Oligosaccharides and their hydrolysis products

Substrate	Hydrolysis Product	Linkage	Enzyme
Maltose	2 Glucose molecules	α-1,4	α-Glycosidase
Sucrose	Glucose, fructose	α-1,2	-
α-Methylglycoside	Glucose, methanol	-	-
Cellobiose	2 Glucose molecules	β-1,4	β-Glycosidase
Raffinose	Galactose, sucrose	α-1,6	α-Glycosidase

Source: Uhlig (1998)

Table 2.6 Polysaccharide and their hydrolysis products

Polysaccharide	Monomer Unit	Linkage	Enzyme
Starch	Glucose	α-1,4	α-amylase or β-amylase
		α-1,6	Isoamylase
Cellulose	Glucose	β-1,4	Cellulase
Dextran	Glucose	α-1,6	-
Xylan	Xylose	β-1,3; β-1,4	Xylanases
Pectin	Galacturonic acid	α-1,4	Pectinase
Araban	Arabinose	α-1,5; α-1,3	Arabinases
Inulin	Fructose	β-1,2	Inulinase

Source: Uhlig (1998)

The specificity of carbohydrases depends on

1) The configuration of the glycosidic linkage.

2) The chemical structure of the monomeric sugars joined by the linkage.

3) The molecular weight of the substrate : α-amylases are able to hydrolyze high-molecular-weight amylose very rapidly, but maltose or maltotriose are hydrolyzed only very slowly, if at all.

2.2.2 Amylase

2.2.2.1 Endoamylases or α-Amylase (EC 3.2.1.1)

The endoamylases hydrolyze the α-1,4 glycosidic linkages in starch, glycogen, and derivatized species. Depending on the source, the properties of

different amylases vary widely. Many α-amylases (α-1,4-glucan-glucanohydrolase) can be produced in pure crystalline form, such as those from malt, pancreas, *Aspergillus oryzae*, and *Bacillus subtillis*. Their properties have been well investigated. They differ in their molecular weights, temperature stability, optimum-activity pH, and hydrolytic specificity. Because of this specificity, different α-amylases produce oligosaccharides or various chain lengths and yields (Uhlig, 1998). The fast and complete degradation of starch requires pregelatinization of the substrate. Bacterial α-amylase degrades gelatinized starch 300 times faster and fungal α-amylase 100,000 times faster than native starch (Walker and Hope, 1963). The rate of hydrolysis depends primarily on the degree of polymerization of the starch, dropping markedly with a lower degree of polymerization. Amylose is hydrolyzed faster than amylopectin (MacGregor, 1988).

Hence, as α-amylolysis proceeds, enzyme action is no longer random, because the proportion of resistant linkage has increased. From amylose, the end products are maltose (about 90%) together with glucose or maltotriose, depending upon the digest condition. From amylopectin, the same end products are produced, together with branched oligosaccharide (α-dextrins) which contain the original interchain linkage. Hence, from a normal starch, the end products are largely maltose, with smaller amounts of glucose or maltotriose, and α-dextrins (Uhlig, 1998).

In contrast to many other starch-degrading enzymes (e.g. β-amylase, pullulanase) α-amylase can attack native starch granules. α-amylolysis can be followed by scanning electron microscopy and reveals the production of cavities

within the granules, during the early stages. Eventually the whole granule may be solubilized (Blanshard and Mitchell, 1979)

2.2.2.2 Exoamylases

The β-amylase or α-1,4-glucan-maltohydrolase (EC 3.2.1.2; CAS 9000-91-3), also known as *saccharogenic* or *maltogenic amylases*, are found in cereals, malted cereals, sweet potatoes, and other plants. β-Amylases hydrolyze malted residues at the α-1,4 linkage from the nonreducing end of the starch chain. The designation does not relate to the configulation of the glycosidic linkage that is hydrolyzed, but rather to the free hydroxyl of the cleavage product: β-maltose. β-amylase does not split α-1,6 linkages. The reaction products from amylopectin are maltose and β-limit dextrin (40 – 45%). The β-amylases occur in different plants as isozymes with various characteristics. Molecular weights vary from 57,000 to 64,000 daltons in cereals and 152,000 daltons for the enzyme in sweet potatoes. The heat stability of β-amylases depends on their origin. When malt, which contains a mixture of α- and β-amylase, was heated to 70°C in the presence of Ca^{2+}, the β-amylase is inactivated. The β-amylase from soybeans was inactivated in 30 min at 70°C (Uhlig, 1998).

The other exoamylase is glucoamylase or α-1,4-glucan-glucohydrolase (EC 3.2.1.3) , it act on starch by splitting glucose units from the nonreducing end. Maltose is broken down only slowly, while 1,6-bonds in the branched polysaccharide are hardly attacked. Thus glucose, maltose and limit dextrins are the end products of glucoamylase action. Microorganisms used to

produce glucoamylase are *Aspergillus niger*, *A. oryzae*, *A. awamori*, *Rhizopus niveus*, and *R. niveus* (Manner, 1979).

2.2.3 Physiology of α-amylase production

The production of α-amylase by submerged fermentation (SmF) and solid state fermentation (SSF) has been thoroughly investigated and is affected by a variety of physicochemical factors. Most notable among these are the composition of the growth medium, pH of the medium, phosphate concentration, inoculum age, temperature, aeration, carbon source and nitrogen source.(Fogarty, and Kelly, 1979; Lonsane, and Ramesh, 1990)

2.2.3.1 Physicochemical parameters

The role of various physico-chemical parameters, including carbon and nitrogen source, surface acting agents, phosphate, metal ions, temperature, pH and agitation have been studied

2.2.3.1.1 Substrate source: induction of α-amylase

α-Amylase is an inducible enzyme and is generally induced in the presence of starch or its hydrolytic product, maltose (Tonomura *et al*, 1961; Morkeberg, Carlsen, and Neilsen, 1995). Most reports available of the

induction of α-amylase in different strains of bacteria and mold. There is a report of a 20-fold increase in enzyme activity when maltose and starch were used as inducers in *Aspergillus oryzae* (NRC 401013) (Eratt *et al*, 1984). Similarly strong α-amylase induction by starch and maltose in the case of *A. oryzae* DSM 63303 has been reported (Lachmund *et al*, 1993). The same results presented in *Bacillus sp*., the enzyme formation with other carbon sources were much lower compared to that with starch and maltose (Santos and Martins, 2003; Mamo and Gessesse, 1999). Strong induction of α-amylase formation has been found in *Xanthomonas campestris pv. campestris*, whereas those grown on starch and maltose showed 53- and 100-fold higher activities, respectively, than those found in glucose grown cells (Tseng and Peng,1985).

The carbon sources as glucose and maltose have been utilized for the production of α-amylase. However, the use of starch remains promising and ubiquitous. A number of other non-conventional substrates as lactose, casitone, fructose, oliseed cakes and starch processing waste water have also been used for the production of α-amylase (Gupta *et al*, 2003)

2.2.3.1.2 Nitrogen source

Organic nitrogen source have been preferred for the production of α-amylase. Yeast extract has been used in the production of α-amylase from various microorganisms. Yeast extract has also been used in conjunction with other nitrogen source such as bactopeptone in the case of *Bacillus sp*. IMD 434 (Hamilton, Kelly, and Fogarty, 1999) and meat extract for *A. oryzae* (Imai, Suzuki, Masamoto, and Nagayasu, 1993). Yeast extract increased the productivity of α-amylase by 110 – 156% in *A. oryzae* when used as an additional

nitrogen source than when ammonia was used as a sole source (Pedersen and Nielsen, 2000). Other organic nitrogen sources have been reported to support maximum α-amylase production by various bacteria and fungi. However, various inorganic salts such as ammonium sulphate for *A. oryzae* (Morkeberg, Carlsen, and Neilson, 1995) and *A. nidulans* (Lachmund, *et al*., 1993), ammonium nitrate for *A. oryzae* have been reported to support better α-amylase production in fungi. However, in microbial production of enzymes, nitrogen can be an important limiting factor (Kole and Gerson, 1989). More than 1% of ammonium sulfate inhibited amylase production in *Bacillus* sp.

Amino acids in conjunction with vitamins have also been reported to affect α-amylase production. However, no conclusion can be drawn about the role of amino acids and vitamins in enhancing the α-amylase production in different microorganisms as the reports are highly variable (Gupta *et al*, 2003).

2.2.3.1.3 Role of phosphate

Phosphate plays an important regulatory role in the synthesis of primary and secondary metabolites in microorganisms and likewise it affects the growth of the organism and production of α-amylase (Dean, 1972). A significant increase in enzyme production in *A. oryzae* above 0.2 M phosphate levels has been reported (Ueno, *et al.*, 1987). Similar result was found in *B. amyloliquefaciens* where low levels of phosphate resulted in severely low cell density and no α-amylase production (Hillier and Wase, 1997). In contrast, high

phosphate concentration inhibited enzyme production by *B. amyloliquefaciens* (Zhang *et al*., 1983).

2.2.3.1.4 Role of other ions

K^+, Na^+, Fe^{2+}, Mn^{2+}, Mo^{2+}, Cl^-, SO_4^{2-} had no effect while Ca^{2+} was inhibitory to amylase production by *A. oryzae* EI 212 (Kundu, Das, and Gupta, 1973). Mg^{2+} played an important role and production was reduced to 50% when Mg^{2+} was omitted from the medium. Na^+ and Mg^{2+} show coordinated stimulation of enzyme production by *Bacillus sp.* CRP strain (Wu, Mabinadji, and Betrand, 1999).

2.3 The *Xanthomonas campestris*

Xanthomonas campestris is a bacteria that causing black rot in crufifers (Schaad and William, 1974). It is also an important microorganism which has been employed to produce exopolysaccharide, xanthan gum, for industrial uses (Sutherland, 1993).

2.3.1 Cell morphology

All *Xanthomonas* cells are Gram-negative rods, sometimes slightly curved with rounded ends, measuring 0.2 – 0.6 μm by 0.8 – 2.9 μm. They occur mostly alone or in pairs but chains and filaments also occur. Exceptionally, cells up to 1.5 μm wide and 4 μm long were found. Cell length is variable, even within the same

strain. The occurrence of pairs of cells is quite typical for *X. maltophilia* and *X. campestris*, but it is not always clear whether it is a pair of cells or an elongated single cell. Most cells have intracellular granules. Polyhydroxyalkanoates may accumulate in low quantities. No spores or other resting states occur (Swing, Vauterin, and Kersters, 1993).

No pili or fimbriae have been reported. Cells are surrounded by xanthan gum (extracellular polysaccharide, EPS), not recognizable as capsules by light or electron microscopy. Cells are motile, sometimes sluggishly. *X. maltophilia* (phenon 1) and *X. albilneans* (phenon 4), which do not form xanthan gum, both have cells that move rapidly. *X. campestris* pv. *graminis* (phenon 8) is non-motile. *X. populi* (phenon 6) contains motile and non-motile strains. Flagella might be difficult to stain by the method described by Rhodes (1958) as in most cases they were easily detached from the cell. A limited number of cells had polar monotrichous flagellation. *X. maltophilia* was multiflagellated.

2.3.2 Growth characteristics

2.3.2.1 Culturing Xanthomonas

Xanthomonas cultures, except *X. populi*, could be grown on GYCA medium, composed of D-glucose (1% w/v), yeast extract (0.5%), $CaCO_3$ (3%) and agar (2%) in screw-tapped tubes. They were incubated for 2 days at 28°C. The working laboratory cultures could be kept at 4°C and must be best transferred monthly (Swing, Vauterin, and Kersters, 1993).

2.3.2.2. Growth temperature

X. populi is unable to grow at temperature above 23°C, whereas all other species grow optimally at 28°C. *Xanthomonas* is unable to grow at 4°C. At 37°C, only a few *X. maltophilia* and *X. campestris* strains are able to grow (Swings, Vauterin, and Kersters, 1993).

2.3.2.3 pH sensitivity

Xanthomonas is not able to grow at an initial pH of 4.5 or lower. At pH 6.5, all *Xanthomonas* strains grow well, except those *X. fragariae*, which require a pH value of 7.5 (Swings *et al*., 1993).

2.3.2.4 Osmo-tolerance

X. campestris and *X. maotophilia* are the only species which tolerate 10% glucose, a few strains even tolerate 20%. *Xanthomonas* cannot grow in 30% glucose. *Xanthomonas* is not very tolerant of NaCl. *X. fragariae* does not tolerate 0.5% NaCl, the lowest concentration tested. Within each individual species, NaCl tolerance may vary between 0.5 and 5% (Swings *et al*., 1993).

2.3.2.5 Oxygen requirement

Xanthomonas is an obligate aerobe and uses oxygen as a terminal electron acceptor. Oxidase activity occurs only in *X. maltophilia* and *X. campestris* strains, but not the other taxa (Rye *et al*., 1988).

2.4 Xanthan gum

2.4.1 Background

Xanthan gum is typically produced copiously as extracellular polysaccharide by the various pathovars of *X. campestris* and by some other *Xanthomonas* species. The production of this exopolysaccharide is manifested in the large mucoid colonies of *Xanthomonas* species on solid media and in the viscosity of liquid cultures (Margaritis and Zajic, 1978).

There has been considerable work on the physiology of xanthan gum production by *X. campestris* and recently a number of the genes involved in xanthan gum synthesis have been identified. Because of its industrial applications and the various unusual properties of the polysaccharide in aqueous solution, xanthan gum has been the subject of a large number of structural and physicochemical studies.

2.4.2 Structure of xanthan gum

The polymer can be readily precipitated from culture broths with polar solvents such as ethanol or isopropanol. Subsequent purification can be achieved by a variety of techniques including fractional precipitation, chromatography and precipitation with quaternary ammonium compounds (Flores-Candia, and Deckwer, 1999).

Acid hydrolysates of xanthan gum contain D-glucose, D-mannose and D-glucuronic acid in the molar ratio 2:2:1. Although other components have been reported in polysaccharides from bacterial isolates described as '*Xanthomonas*' species, the strains involved have generally been poorly characterized. Isolated polysaccharide from several of the other *Xanthomonas* species seems to share the same composition as that from pathovars of *X. campestris*. The primary structure of the polysaccharide is composed of a pentasaccharide repeat unit. This is effectively a main cellulose chain to which trisaccharide side-chains are attached at the C-3 position on alternate D-glucosyl residues (Jansson, Kenne, and Lindberg 1975; Menton *et al*., 1976). Depending on the bacterial strain and on the physiological conditions for bacterial growth, the polysaccharide may carry varying amounts of O-acetyl groups on the C-6 position of the internal α-D-mannosyl residue ane 4,6-carboxyethylidene (pyruvate ketal) on the side-chain terminal β-D-mannosyl residue, respectively (Sandford and Baird, 1983).

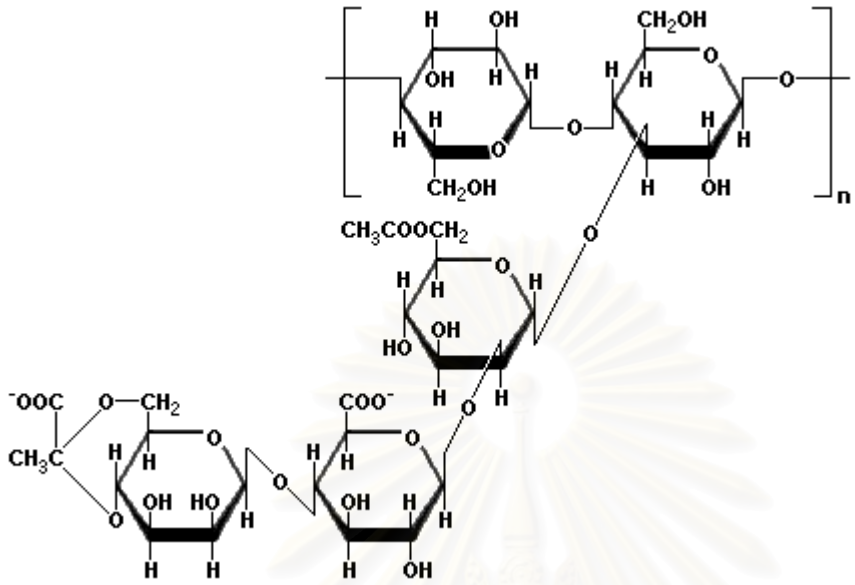


Fig 2.1 Structure of xanthan gum (Baird and Pettitt, 1991)

2.4.3 Production of xanthan gum

Xanthan gum is produced by fermentation, using a pure culture of *Xanthomonas campestris* with glucose as the substrate, followed by purification and recovery with alcohol (Rocks, 1971).

The process for making xanthan gum is shown in Fig 2.2. First, the selected microbial strain is preserved for possible long-term storage by proven methods to maintain the desired properties. A small amount of the preserved culture is expanded by growth on solid surfaces of in liquid media to obtain the inoculum for large bioreactors. The growth of the microorganism and xanthan gum production are influenced by factors (Garcia-Ochoa *et al*., 2000) such as the type of bioreactor used, the mode of operation (batch and continuous), the medium composition, and the culture conditions (temperature, pH, dissolved oxygen concentration).

At the end of fermentation, the broth contains xanthan gum, bacterial cell, and many other chemicals. For recovering the xanthan gum, the cells are usually removed first, either by filtration or centrifugation. Further purification may include precipitation using water-miscible non-solvents (isopropanol, ethanol, acetone), addition of certain salts, and pH adjustments. The FDA regulation for food grade xanthan gum prescribed the use of isopropanol for precipitation. After precipitation, the product is mechanically dewatered and dried. The dried product is milled and packed into containers with a low permeability to water (Flores-Candia and Deckwer, 1999).

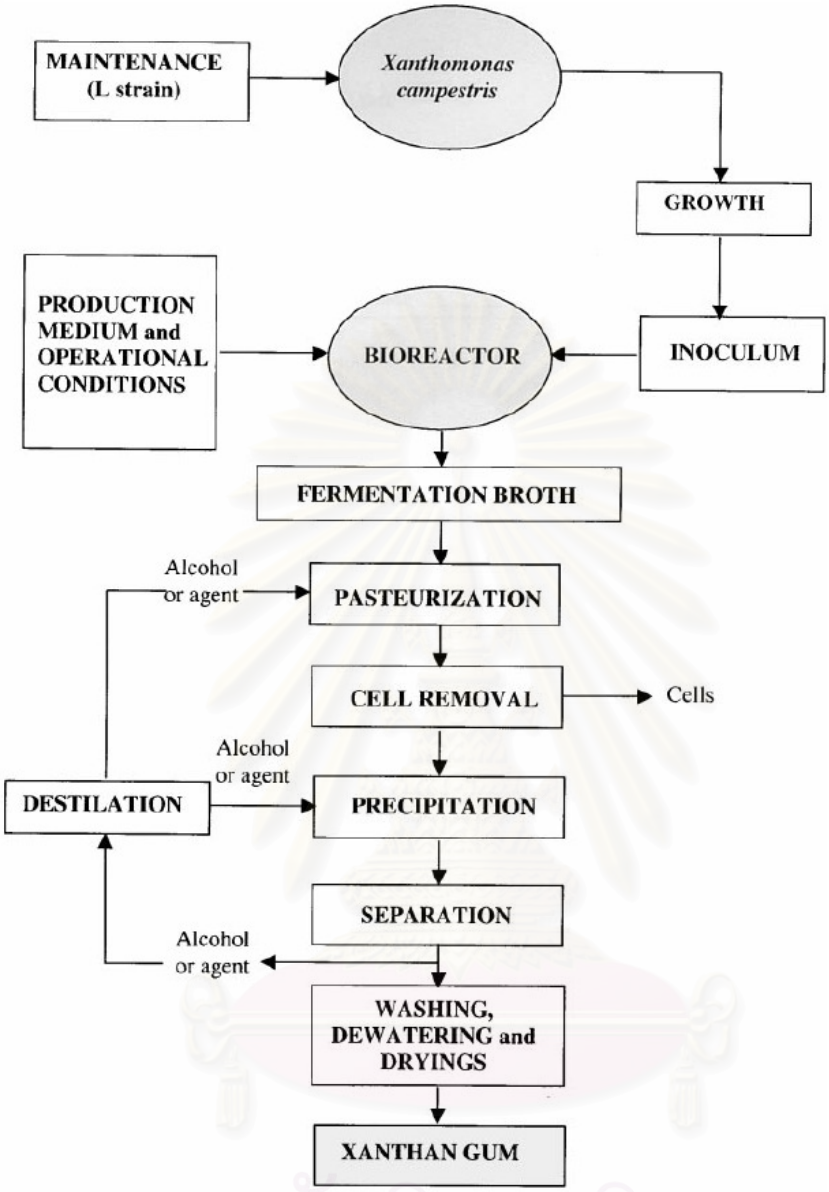


Fig 2.2 Outline of the xanthan gum production process (Garcia-Ochoa *et al*., 2000)

2.4.3.1 Production medium

To produce xanthan gum, *Xanthomonas campestris* needs several nutrients, including macronutrients such as carbon and nitrogen and micronutrients such as potassium, iron, and calcium salts (Cadmus *et al*., 1978)

2.4.3.1.1 Carbon and nitrogen source

Glucose and sucrose are the most frequently used carbon source. The concentration of carbon source affects the xanthan gum yield, 2 – 4% is preferred (Souw and Demain, 1979). Higher concentration of glucose and sucrose inhibit growth of *Xanthomonas campestris*. Funahashi *et al*. (1987) reported growth limitation accompanied by a large decrease in gum production at initial glucose concentrations higher than 50 g/l. Therefore, several reports suggest that higher xanthan gum production can be obtained by controlling the carbon : nitrogen (C:N) ratio in both growth and production phases of fermentation (De Vuyst, Van Loo, and Vandamme, 1987; Funahashi *et al*., 1987). It is generally considered that a low C:N ratio is required in cell growth phase to achieve both high cell concentration as well as high specific growth rates, which in turn can result in higher xanthan gum production. On the other hand, xanthan gum biosynthesis is favored by high C:N ratio.

Although, glucose and sucrose are known as the best carbon source for xanthan gum productions. There are many researchers tried to produce xanthan gum by less expensive substrate to reduce price of this famous

polysaccharide. In 1958, Lilly, Wilson, and Leach reported that *Xanthomonas campestris* could grown and produced polysaccharide in medium contained sugar and starch, such as glucose, sucrose and soluble starch from corn starch. Sorghum flour (Weber and Horam, 1966), dry-milled cornstarch (Miescher and Haute 1969), and corn flour (Duc *et al.*, 1978) were used as carbon source for xanthan gum production but glucose and sucrose are still the best substrates. Several reports explored the effect of agricultural waste and wastewater as substrate for xanthan gum production (Yoo and Harcum, 1999; Lopez, Moreno, and Ramos-Cormenzana, 2001; Lopez *et al*., 2004). They indicated that type, such as cellulose and pectin, and amount of carbon in the media were the important factors for yield and properties of xanthan gum.

It is generally believed that a high nitrogen concentration is required for fast cell growth and high cell density of *Xanthomonas campestris*, whereas xanthan gum biosynthesis is favored by a high concentration ratio of carbon source and nitrogen source (Garcia-Ochoa, Garcia-Leon, and Romero, 1990). The specific rate of xanthan gum production depended on concentration of nitrogen source, not on type of it (Pinches and Pallent, 1986). However, Souw and Demain (1979) reported that excess concentration of effective nitrogen compounds strongly inhibited xanthan gum production and xanthan gum production is best under nitrogen limitation. The same result reported by De Vuyst and Vermeire (1994), and Godet (1973). Nitrogen is very important on growth of bacteria but not for xanthan gum production (Amanullah, Satti and Nienow, 1998). There were many reports showed the increasing of cell yield and specific growth rate as C:N ratio decreased (Lo, Yang, and Min, 1997; and Rajeshwari, Prakash, and Ghosh, 1995).

Nitrogen concentration control in growth phase and xanthan gum production period can increase xanthan gum yield (De Vuyst and Vermeire, 1994). Initial nitrogen concentration is also influence the xanthan gum molecular structure and properties. Initial nitrogen concentration does not show any influence on xanthan gum average molecular weight but effect on pyruvate content. Casas, Santos, and Garcia-Ochoa (2000) reported that, when nitrogen concentration increases, pyruvate content decreases, and this is in agreement with results previously reported by Davidson (1978).

2.4.3.1.2 Mineral source

The mineral source is the cofactor and composition of coenzyme in bacterial metabolism. Roseiro *et al*. (1992) reported that Ca^{2+} proved to be a major factor affecting the increase of viscosity of xanthan gum production broth. Phosphorus nutrition also appears to be important in the xanthan gum fermentation. Souw and Demain (1979) studied on the effect of inorganic phosphate on xanthan gum production and their results indicated that xanthan gum production was controlled by phosphate, but phosphate concentration higher than the optimum level (50 mM) suppressed xanthan gum production. In 1994, De Vuyst and Vermeire reported that increasing concentration of inorganic phosphate resulted in a decrease in xanthan gum formation and also in a lower pyruvate content of the xanthan gum produced. Moreover, De Vuyst and Vermeire reported that the quality of xanthan gum produced mainly depends on its pyruvate content and citrate was

believed to have a positive effect on it. They found that the pyruvate content of the xanthan gum formed increased with increasing citrate concentration.

2.4.3.2 Production conditions

2.4.3.2.1 Inoculum build-up

During inoculum buildup the aim is to increase the cell concentration but minimize the production of xanthan gum because xanthan gum around the cells impedes mass transport of nutrients and extends lag phase of growth (Silman and Rogovin, 1970). In 1995, Harding, Cleary, and Ielpi reported that xanthan gum is the secondary metabolite while *X. campestris* produce at the end of log phase to the stationary phase of growth. So the inoculum should be grown between the middle of log phase and the stationary phase that is 24 – 48 h. The suitable inoculum volume for the production bioreactor is between 5 and 10% of total broth volume in the vessel. (Pinches and Pallent, 1986).

2.4.3.2.2 Temperature

Temperature employed for xanthan gum production range from 25 to 34°C, but culture at 28°C and 30°C is quite common. The influence of temperature on xanthan gum production has been widely studies. Thonart *et al*. (1985) reported an optimum process temperature of 33°C, proposing a temperature of 25°C for growth and 30°C for production. Shu and Yang (1990) claimed that the

optimal temperature depended on the final objective. For a high xanthan gum yield, the temperature between 31 and 33°C was recommended, but culture at 27 to 33°C was better at attaining a high pyruvate content in xanthan gum.

2.4.3.2.3 pH

Most authors agree that neutral pH is the optimum value for growth of *Xanthomonas campestris.* During xanthan gum production, the pH decreases from neutral to values close to 5 because of acid groups present in xanthan gum. Some authors suggest that pH control is not necessary for this process but others recommend control at neutral pH (Rocks, 1971). Garcia-Ochoa, Santos, and Alcon (1996) reported that when pH was controlled, xanthan gum production ceases once the stationary phase was attained but when pH was not controlled the gum production continuous during the stationary phase.

2.4.3.2.4 Oxygen transfer rate

Various type of bioreactors have been used to produce xanthan gum, but stirred tank is employed the most frequently. In stirred bioreactors, the rate of oxygen transfer is influenced by the air flow rate and stirred speed. When stirred tanks are used, air flow rate is generally maintained at a constant value, usually not more than 1 l/l min. In contrast, the agitation speed used varies over broad range. Garcia-Ochoa *et al.* (1997) found that when the stirred speed was constant at less than 500 rpm, the production of xanthan gum was reduced because

oxygen mass transfer become limiting with increasing viscosity of the broth. But when stirred speed was constant at higher than 500 rpm, xanthan gum production was also poor because the cells were affected by the mechanical agitation. Similar results have been reported by Moo-Young, Chiti, and Vlach (1993).

2.4.4 Properties of xanthan gum

2.4.4.1 Pseudoplastic gum

Solutions of xanthan gum are pseudoplastic. Xanthan gum dissolves readily in hot or cold water to give high viscosity solution at low concentration. The viscosity of a 1% solution is about 800-1,000 cps when measured at 60 rpm on a Model LVF Brookfield Viscometer (Rocks, 1971). Flow properties of most materials are described as Newtonian, thixotropic, or pseudoplastic. Most systems are Newtonian. In these system, stress increases in a linear manner with shear rate. Because of its pseudoplastic nature, xanthan gum is an excellent suspending agent (Sutherland, 1994). The minimum shear effect of mastication is sufficient to temporarily break the viscosity of xanthan gum solution. This pseudoplastic property is also of tremendous value when a thickened system must be pumped within a food plant.

2.4.4.2 Stability of xanthan gum

2.4.4.2.1 Temperature

Most aqueous gum solutions show a sharp decrease in viscosity with increase in temperature. If the high temperature is maintained for only a short period of time, these solutions will usually regain their original viscosity when they are brought back to room temperature. Solutions of xanthan gum, on the other hand, have the remarkable property of maintaining their viscosity regardless of changes in temperature. Products containing xanthan gum will have excellent stability over a wide temperature range. Solutions of xanthan gum can be held at elevated temperature for long periods of time without any major change in viscosity. For example, only minor viscosity changes are observed when solutions are autoclaved at 121°C for 15 – 30 minutes. There also appears to be little effect when xanthan gum solutions are subjected to long exposures to temperature around 80°C (Rocks, 1971).

The presence of salts improves the resistance of xanthan gum to degradation by heat. Most food products normally contain sufficient salts to give excellent heat stability. This property of xanthan gum makes it attractive for use in various canned products, as well as in gravies and sauces that might be kept hot on a steam table for several hours (Garcia-Ochoa *et al*., 2000).

Xanthan gum solutions are also quite stable under freezing condition. At very low levels, on the order of 0.1 – 0.2%, xanthan gum contributes outstanding

freeze-thaw stability to many food products which also contain starch, such as pudding and spoonable-type dressings.

2.4.4.2.2 pH

Many water soluble gums change markedly in viscosity as the pH of their solutions is changed. In fact, many of them are unstable at pH extremes, particularly on the acid side. In contrast, the viscosity of aqueous solutions of xanthan gum is nearly independent of pH changes. Xanthan gum will dissolve directly at any pH that might be encountered in a food product. In the industrial area, xanthan gum is being used as a thickener in solutions containing 8% hydrochloric acid of 5% sodium hydroxide (Rocks, 1971).

2.4.5 Applications of xanthan gum

The unique physical and rheological properties of xanthan gum make it one of the most versatile hydrocolloids for use in a host of food, pharmaceutical and personal care products. These properties result in products with longer shelf life, improved flow, consistent viscosity, better texture, and a pleasing appearance (Baird and Pettitt, 1991). Xanthan gum was very useful for several food products. Ferrero, Martino and Zaritzky (1993) found that the addition of xanthan gum into starch paste could minimize spongy structure formation in precooked frozen food. The addition of xanthan gum increased both water-holding capacity and elastic modulus of microwave assisted freeze-drying product (Barrett, *et al.*, 1997). A mixture of

xanthan gum, locust bean and guar gum was found to give the best textural properties and melting quality of a soy-gelatin mozzarella cheese analog (Yang, Taranto, and Cheryan, 1983)

2.4.5.1 Food applications (Rocks, 1971)

1) Xanthan gum at low levels provides high viscosity, which is stable to changes in temperature, in sauce and gravies at both acid and neutral pH.

2) In starch-based desserts, such as puddings, mousses, and flans, inclusion of xanthan gum provides additional body of structure, improved mouthfeel, and reduced syneresis up on storage.

3) Baked goods containing xanthan gum often show increased volume and have improved eating quality.

4) The inclusion of xanthan gum in dry mix bases for beverages provides a pleasant body and mouthfeel to the reconstituted drink.

5) Blends of xanthan gum, carrageenan, and galactomannans are excellent stabilizers for frozen and chilled dairy products, including ice cream, sherbert, and sour cream.

6) The stability of xanthan gum to acid and salt, its effectiveness at low concentrations, and its highly pseudoplastic solution rheology make it the ideal choice for stabilization of no-oil, and regular-oil dressings.

2.5 Chemical mutation

2.5.1 Background of mutation

The term of mutation refers to both the change in the genetic material and the process by which the change occurs. An organism that exhibits a novel phenotype resulting from a mutation is called a mutant. Today, the term mutation often is used in a narrow sense to refer only to changes occurring within genes. Mutation is the ultimate source of all genetic variations, it provides the raw material for evolution. Mutations occur in all genes of all living organism. These mutations provide new genetic variability that allows organism to adapt to environmental changes. Mutation can occur both spontaneous and inducible. The spontaneous mutations are those that occur without a known cause. They may truly be spontaneous, resulting from a low level of inherent metabolic errors, or they may actually be caused by unknown agents present in the environment. The induced mutations are those resulting from exposure of organism to physical and chemical agents that cause changes in DNA (or RNA). Such agents are called mutagents, they include ionizing irradiation, ultraviolet light, and a wide variety of chemicals (Drake, 1970).

2.5.2 Mutation induced by chemical

The discovery of chemical mutagens with known effects on DNA led to a better understanding of mutation at the molecular level. Many chemical are mutagenic. Mustard gas (sulfur mustard) was the first chemical shown to be mutagenic.

Chemical mutagents can be divided into two group: (1) those that are mutagenic to both replicating and non replicating DNA, such as the alkylating agent and nitrous acid and (2) those that are mutagenic only to replicating DNA, such as

base analogs-purines and pyrimidines with structures similar to the normal base in DNA (Russel, 1996).

2.5.2.1 Base analogs

The mutagenic base analogs have structures similar to the normal bases and are incorporated into DNA during replication. However, their structures are sufficiently different from the normal bases in DNA that they increase the frequency of mispairing and thus mutation during replication. The two most commonly used base analogs are 5-bromouracil and 2-aminopurine (Russel, 1996).

2.5.2.2 Alkylating agents

These chemicals react directly with certain bases and thus do not require active DNA synthesis in order to act but still do require DNA synthesis in order to be "fixed". They are very commonly used because they are powerful mutagens in nearly every biological system. Examples of alkylators include ethyl methanesulfonate (EMS), methyl methane sulfonate (MMS), diethylsulfate (DES), and nitrosoguanidine (NTG, NG, MNNG) as shown in figure 8. These mutagens tend to prefer Guanine-rich regions, reacting to form a variety of modified G residues, the result often being depurination. Some of these modified G residues have the property of inducing error-prone repair, although mispairing of the altered base might also be possible. This stimulation of error-prone repair allows all sorts of mutation types to occur as a result of these mutagens, though base substitutions are by far the most frequent. It also appears that alkylated bases can mispair during

replication (Snustad and Simmons, 2000). The relative contribution of all of these mechanisms to actual mutagenesis is unclear.

2.5.2.3 Other chemical mutagents

Nitrous acid is another chemical mutagen that causes oxidative deamination of particular bases. It converts adenine to hypoxanthine (which now pairs with C), cytosine to uracil (which now pairs with A) and finally guanine to xanthine (which still continues to pair with C). Unlike the above mutagens, nitrous acid alters a base directly to a "miscoding" form and thus does not require subsequent DNA synthesis for its effect (Russel, 1996).

CHAPTER III

MATERIALS AND METHODS

3.1 Material

Cassava starch, Dragon Fish brand, from Thai Thum Industrial.

3.2 Apparatuses and Chemical Reagents

3.2.1 Apparatuses

Name	Model	Country
Autoclave	TOMY SS-325, Hiclave HVA-85	USA
Bioreactor	B. Braun Biostat B	Germany
Centrifuge	Hettich Universal 32 R	Germany
CHNS/O Analyzer	PE-2400	USA
Colony counter	Gallenkamp	Germany
Hot Air Oven	Memmert	Germany
Incubator	BMT, Incucell	USA
HPLC apparatus	Water	USA
Incubator Shaker	New Brunswick Scientific	USA
Laminar Flow	ISSCO BVT12	USA
Magnetic Stirrer	Sternam Steroglass	USA

Name	Model	Country
Microfiltration Apparatuses	-	-
pH Meter	Qis M360	Germany
Scale (3 points)	Satorius BP 221S	Germany
Scale (4 points)	Mettler Toledo AB204	USA
Scanning Electron Microscope	JSM-6400	USA
Spectrophotometer	Spectronic Genesys 20	USA
Ultrafiltration Apparatuses	-	-
Water Bath Shaker	Julabo SW23	Germany
Vacuum Pump	Gast Manufacturing Inc.	USA

3.2.2 Chemical reagents

Name	Company	Country
Agar Powder	Himedia	India
Ammonium Sulphate	Univar	Australia
Boric Acid	Univar	Australia
Calcium Carbonate	Univar	Australia
Calcium Chloride	Univar	Australia
Casein from Bovine Milk	Fluka	India
Citric Acid	Univar	Australia
3,5-Dinitrosalicylic Acid	Himedia	India
D-glucose	Univar	Australia

Name	Company	Country
Ethyl Methansulfonate	Fluka	India
Hydrochloric Acid	BDH	UK
Iron (III) Chloride	Unilab	Australia
Iron (III) Sulphate	Univar	Australia
Magnesium Chloride	Univar	Australia
Magnesium Sulfate	Univar	Australia
Malt Extract	Himedia	India
Manganese Chloride	Carlo Erba	USA
Peptone	Himedia	India
Phenol Detached Crystals	Fisher Scientific	UK
di-Potassium Hydrogen Orthophosphate	Univar	Australia
Potassium di-Hydrogen Phosphate	Univar	Australia
Potassium Iodate	Univar	Australia
Potassium Iodide	BDH	UK
Potassium Sodium Tartrate	Univar	Australia
Silicone Antifoaming Agent	BDH	UK
Sodium Hydroxide	Carlo Erba	USA
Sodium Thiosulphate	Univar	Australia
Starch, Soluble	Univar	Australia
Sulfuric Acid	J.T. Baker	USA
tri-Sodium Citrate	Univar	Australia
Yeast Extract	Himedia	India
Zinc Oxide	Univar	Australia

3.3 Microorganism

Xanthomonas campestris TISTR 840 was obtained from Thailand Institute of Scientific and Technological Research.

3.4 Storage of microorganism and the medium

The organism was maintained on YM agar (Appendix A-1). Cells were incubated at 30°C for 24 h and then stored at 4°C to use as the starter. Subculture was done every 14 days.

3.5 Preparation of *X. campestris* starter

One loop of *X. campestris* from 3.4 was transferred into 100 ml of YM broth and incubated at 30°C, 200 rpm, for 24 h. The cultured broth, 10 ml was then transferred to new fresh 100 ml of YM broth. Cells were incubated at 30°C, 200 rpm for 24 h to use as starter culture.

3.6 Methodology

3.6.1 Effect of cassava starch on xanthan gum production by X. campestris TISTR 840

3.6.1.1 Effect of gelatinized and raw cassava starch on xanthan gum production

Starter culture from 3.5 was placed into 5 liters bioreactor (Fig 3.1) with 3 liters XOL basal medium (Appendix A-3), plus 0.125% peptone (Tseng and Peng, 1985, using 1.0% of gelatinized and raw cassava starch as a carbon source. The raw cassava starch was sterilized at 105°C for 12 h in dry oven (Hoshino *et al.*, 1990) and mix with sterilized XOL medium. The gelatinized cassava starch medium was done by mixed raw cassava starch in 3 liters of XOL basal medium and heated to 80°C in water bath then sterilized in a 5 liter reactor. Cells were incubated at 30°C, pH 7.0 ± 0.1, aeration rate 0.5 vvm and stirred at 200 rpm. The culture medium was collected at 120 h to measure the crude xanthan gum content (Appendix B-4).

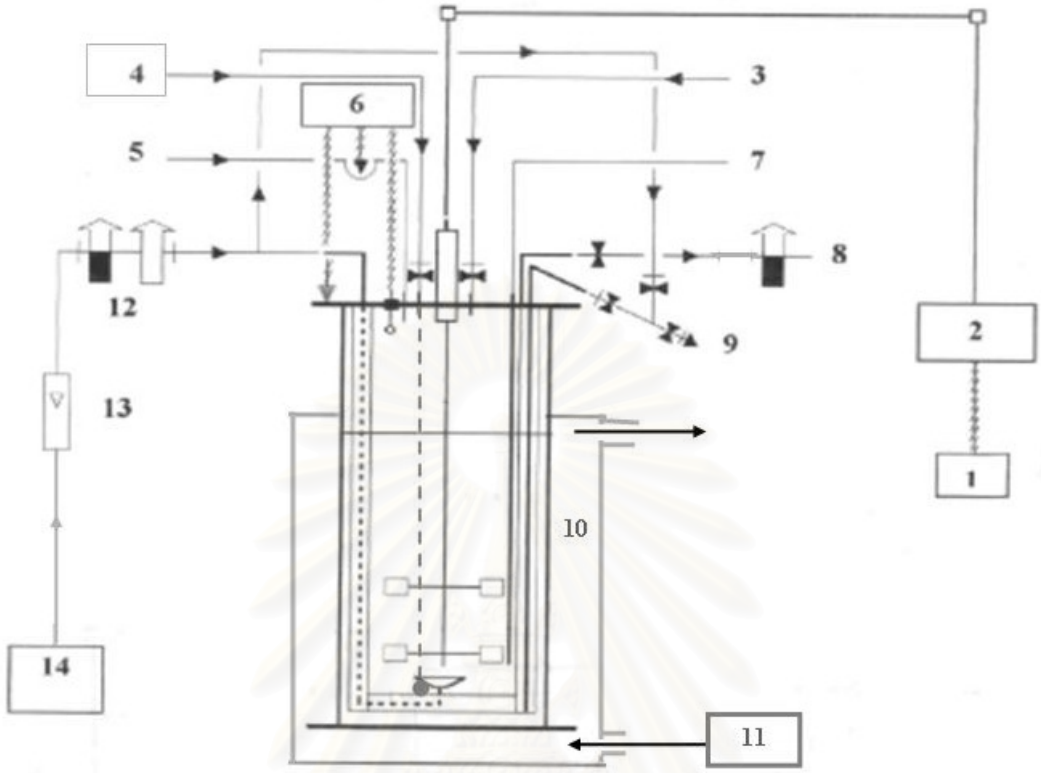


Fig 3.1 Diagram of the bioreactor: (1) engine regulator, (2) engine, (3) inoculum, (4) pH controller, (5) antifoam, (6) antifoam controller, (7) thermometer, (8) air exhaust system with air filter, (9) sampling, (10) water jacket, (11) temperature controller, (12) air filter, (13) flow meter, (14) air pump.

3.6.1.2 Determination of xanthan gum purity

The commercial food grade xanthan gum (Thai Food and Chemical Co., Ltd.) and crude xanthan gum produced from gelatinized and raw cassava starch were dissolved to the 0.2% concentration in sodium hydroxide solution pH 11. The mixture was then filtered through the 0.45 µm nylon filter. The molecular weight of xanthan gum was determined by gel permeation chromatography using Ultrahydrogel Linear column. The sodium hydroxide

solution, pH 11, was used as mobile phase with flow rate 0.6 ml/min. Infrared detector was used to detect the sample and the results was compared with pullulan at various molecular weight 5,500 to 788,000 Dalton which used as standard plot.

3.6.2 Effect of raw cassava starch on amylase production by X. campestris TISTR 840

The starter culture from 3.5 was placed into a 5 liter bioreactor with 3 liters XOL basal medium, plus 0.125% peptone, containing 1.0% raw cassava starch. Cells were incubated at 30°C, pH 7.0 ± 0.1, aeration rate 0.5 vvm and stirred at 200 rpm for 32 h. The bacterial culture was collected every 4 h. The cell growth was determined by measuring the absorbance at 565 nm. The amylase activity and reducing sugar content in culture media were determined by DNSA method (Appendix B) presented by Robyt and Whelan (1965).

3.6.3 Partial purification and characterization of amylase produce by X. campestris TISTR 840

3.6.3.1 Partial purification of amylase produced by X. campestris

Purification steps were modified from report of Tseng and Peng (1985). After 16 h cultivation of *X. campestris*, the fermentation broth was centrifuged at 10,000 x g at 4°C for 15 minute to remove the cell. The supernatant was filtered through a microfiltration membrane, pore size 0.45 μm. The filtrate was

concentrated with an ultrafiltration membrane, pore size 10,000 Dalton, and then dialyzed against 10 mM potassium phosphate buffer (pH 7.0). The solution was lyophilized and resuspended in small volume of distilled water and used as partial purification sample of amylase produced from *X. campestris*.

3.6.3.2 Effect of temperature on partial purified amylase

The temperature activity profile of the partial purified amylase was determined over the range 30 – 75°C by adding 2.0 ml of the partial purified enzyme preparation (0.514 U/ml) to 0.5 ml of 1% soluble starch suspended in 0.05 M potassium phosphate buffer, pH 7.0, and incubating for 30 min at the test temperature. The mixture (1.0 ml) was collected and the reaction was stop by adding 1 ml of DNSA and activity of amylase was assayed by DNSA method (Appendix B). This method was modified from report of Malhotra, Noorwez, and Satyanarayana (2000).

3.6.3.3 Effect of pH on partial purified amylase

The effect of pH on the activity of partial purified amylase was studied over the pH range 3.0 – 8.0 using McIlvaine's buffer (Appendix A). The reaction mixture comprised 1.0 ml of the partial purified enzyme preparation (0.514 U/ml) and 2.0 ml of 1% soluble starch suspension in appropriate buffer at pH 3.0 – 8.0. The mixture was incubated for 30 min at 35°C and the amount of reducing

sugar released was assayed by DNSA method (Appendix B). This method was modified from report of Tseng and Peng (1985).

3.6.3.4 Effect of ions on partial purified amylase

To determine effect of ions on partial purified amylase, the method form report of Tseng and Peng (1985) was modified. $FeSO_4$, $MgCl_2$, $CaCl_2$ and $MnCl_2$ at 5.0 mM of final concentration were included in the reaction mixture that contained the partial purified amylase and 1.0% soluble starch in 50 mM potassium phosphate buffer (pH 7.0). The mixtures were incubated at 35°C for 30 min and amylase activities were determined by DNSA method (Appendix B).

***3.6.4 Effect of peptone on growth and amylase production of X. campestris* TISTR 840**

Starter culture from 3.5 was placed into 5 liters reactor with 3 liters XOL basal medium, with and without 0.125% peptone, containing 1.0% of gelatinized cassava starch as carbon source. Cells were incubated at 30°C, pH 7.0 ± 0.1, aeration rate 0.5 vvm, and stirred at 200 rpm for 32 h. Culture was collected every 4 h and measured the enzyme activity following cell growth. The cell growth was determined by measuring the absorbance at 565 nm., amylase activity, and reducing sugar content in culture media was detected by DNSA method (Appendix B).

Completely Randomized Design was used as statistical analysis for 3 replicates. Duncan's new multiple range test was used to explain the difference of factors.

3.6.5 Effect of C:N ratio and aeration rate on amylase and xanthan gum production

The percentage of carbon and nitrogen of cassava starch and peptone were analyzed by CHNS/O Analyzer to calculate C:N ratio in this experiment. The fermentation of *X. campestris* TISTR 840 was carried in 5 liters reactor with 3 liters XOL basal medium plus 0.125% of peptone. Raw cassava starch and ammonium sulfate were used as carbon and nitrogen source, respectively, and C:N ratio was varied at 10:1, 20:1, and 30:1. The starter culture from 3.5 was placed into the cultured medium and incubated at 30°C, pH 7.0 ± 0.1, aeration rate was varied for 0.0, 0.5 and 1.0 vvm and stirred at 200 rpm for 32 h. Culture was collected every 4 h and measured the enzyme activity following cell growth with methods as described in 3.6.2. The culture medium was collected again at 120 h to measure the crude xanthan gum content (Appendix B).

Central Composite Design, 2 factors and 3 levels, was used as statistical analysis for 4 replicates at the centre point (Alves *et al.*, 1998). The experimental design was showed in Table 3.1. The obtained results were used to create quadratic equations to determine the correlation of data by SPSS program version 13 and to make response surface curves ($p \leq 0.01$) and a contour plot by STATISTICA program version 6.5.

Table 3.1 Experimental design for amylase and xanthan gum production

Run	C:N ratio	Aeration rate (vvm)
1	10:1 (-1)	0.0 (-1)
2	30:1 (+1)	0.0 (-1)
3	10:1 (-1)	1.0 (+1)
4	30:1 (+1)	1.0 (+1)
5	10:1 (-1)	0.5 (0)
6	30:1 (+1)	0.5 (0)
7	20:1 (0)	0.0 (-1)
8	20:1 (0)	1.0 (+1)
9	20:1 (0)	0.5 (0)
10	20:1 (0)	0.5 (0)
11	20:1 (0)	0.5 (0)
12	20:1 (0)	0.5 (0)

3.6.6 Overproduction of amylase in X. campestris TISTR 840 and its effect on xanthan gum production in raw cassava starch medium

3.6.6.1 Overproduction of amylase in X. campestris TISTR 840 by ethyl methansulfonate (EMS)

The EMS treatment was modified from that described by Miller (1992). *X. campestris* was grown at 30°C in 100 ml YM broth to the OD_{565} of 1.00 $\pm$ 0.10. Culture (5 ml) was centrifuged at 8,000 rpm for 15 min, washed twice with "A buffer" (Appendix A-5) and resuspended in 2.5 ml of the same buffer. The cell suspensions were dispensed as 2 ml aliquots into screw-capped tubes. Then 40 µl of EMS was added to each tube, and incubated at room temperature (28 - 31°C) with mild shaking for 10 to 60 min. To quench the mutagen, 4 ml of a freshly prepared 10% (w/v) sodium thiosulfate sterile solution was added to each tube. The treated cells were then centrifuged at 8,000 rpm for 15 min, washed twice in minimal A buffer and resuspended in 2 ml of the same buffer. Each suspension was diluted in appropriate dilution and spread on YM agar and incubated at 30°C for 48 h. Finally, the survival curve was created and the time gave 10% of survival was selected and picked up every single colony to test ability of amylase production in starch agar plate. The colony gave a large clear zone in starch agar plate was collected and cultured in XOL medium plus 1% raw cassava starch to test xanthan gum production ability (Kamal *et al*., 2003). The selected isolate was cultured and stored at -20°C in 30% glycerol XOL medium.

3.6.6.2 Effect of overproduction of amylase in X. campestris TISTR 840 on xanthan gum production

Wild-type and mutant strain selected from 3.6.6.1 were cultured in 5 liters bioreactor with 2 liters XOL basal medium plus 0.125% of peptone. Raw cassava starch and ammonium sulfate was used as carbon and nitrogen source, respectively, at C:N ratio 30:1. Cells were incubated at 30°C, pH 7.0 ± 0.1, aeration rate 0.5 vvm and stirred at 200 rpm for 120 h. Culture was collected every 4 h and measured the enzyme activity, as described in 3.7, following cell growth, using YM plate count. The amount of xanthan gum was detected during time by method descript in Appendix B

Completely Randomized Design was used as statistical analysis for 3 replicates. Duncan's new multiple range test was used to explain the difference of factors.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Effect of cassava starch on xanthan gum production by *X. campestris* TISTR 840

Xanthan gum is an extracellular heteropolysaccharide produced by the bacterium *Xanthomonas campestris* (López *et al.*, 2004). The excellent rheological properties of xanthan gum contributed to its wild-range of applications as a suspending, stabilizing, and thickening agent in the food industry and its used as an emulsifier, lubricant, thickening agent and mobility control agent to enhance oil recovery (Margaritis and Pace, 1985). Currently, the worldwide consumption of xanthan gum was more than 25 million kg/y, and increased continuously (Yoo and Harcum, 1999). Commercially available xanthan gum was relatively expensive due to glucose or sucrose being used as the sole carbon source.

4.1.1 Effect of gelatinized and raw cassava starch on xanthan gum production

Several researchers had investigated using less expensive carbon sources to produce xanthan gum (Bilanovic, Shelef and Green, 1994; Lopez and Ramos-Cormenzana, 1996; Papi *et al*., 1999; López *et al.*, 2004). Because of the availability

of cheap cassava starch in Thailand, this experiment used the XOL minimal medium containing gelatinized cassava starch as a sole carbon source to produce xanthan gum. The result showed that this substrate could use to produce xanthan gum (Table 4.1) and xanthan gum production was higher than using glucose as a carbon source. This production was too high because the bacterium had to digest the cassava starch before took the hydrolyzed products in to the cell. Because of the hydrolyzing of gelatinized cassava starch could not complete as a report of gelatinized cassava starch that hydrolyzed by new generation of amylase produced from *Aspergillus kavachi* showed the highest DE at 50% after 24 h of hydrolysis (Shariffa *et al*., 2009). From this reason, 1% gelatinized cassava starch used in our fermentation system should produce xanthan gum at lower than 6.99 g/l.

Table 4.1 Effect of carbon source on xanthan gum production

Carbon source	Xanthan gum (g/l)
1% Gelatinized cassava starch	$9.06^{a} \pm 0.12$
1% raw cassava starch	$2.23^{b} \pm 0.04$
1% glucose*	6.99

$^{a,b,\cdots}$ is the significantly difference ($p \leq 0.05$) with 3 replications of each treatment

* Psomas, Liakopoulou-Kyriakides, and Kyriakidis (2007)

From the result, xanthan gum production by gelatinized cassava starch might has impurity such as the remaining gelatinized starch that precipitated with 95% ethyl alcohol as same as xanthan gum.

4.1.2 Determination of xanthan gum purity

The results from Table 4.1 indicated that crude xanthan gum produced from gelatinized cassava starch might include with high impurity of the small molecular weight substances, such as unhydrolyzed starch, because cassava starch had amylopectin that is highly branches and could not hydrolyze by amylase (Whistler, BeMiller, and Paschall, 1984). The impurities dissolved in the cultured medium and they were precipitated with 95% ethyl alcohol as same as xanthan gum. The size exclusion HPLC was used to prove this hypothesis and the results were showed in Fig 4.1 and 4.2.

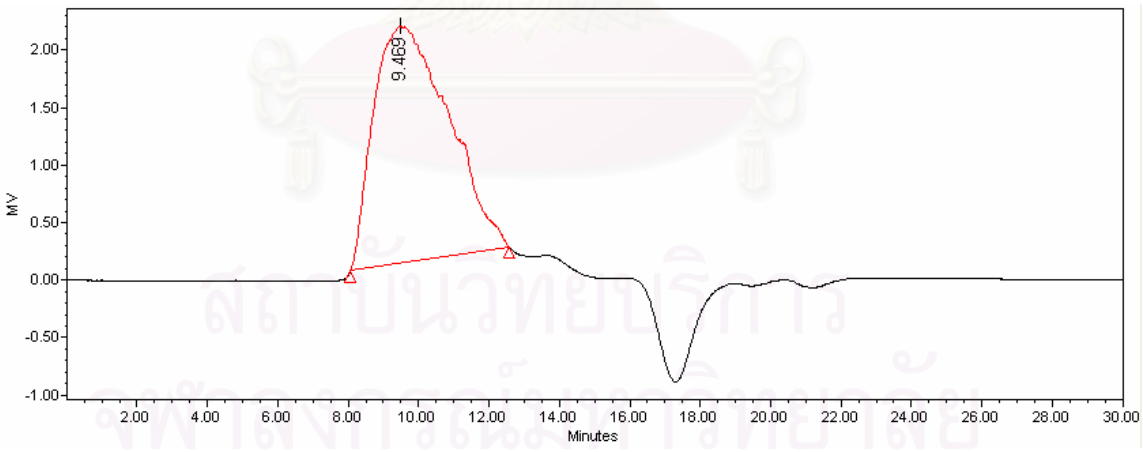


Fig 4.1 Size exclusion HPLC chromatogram of 1% commercial food grade xanthan gum from Thai Food and Chemical Co., Ltd.

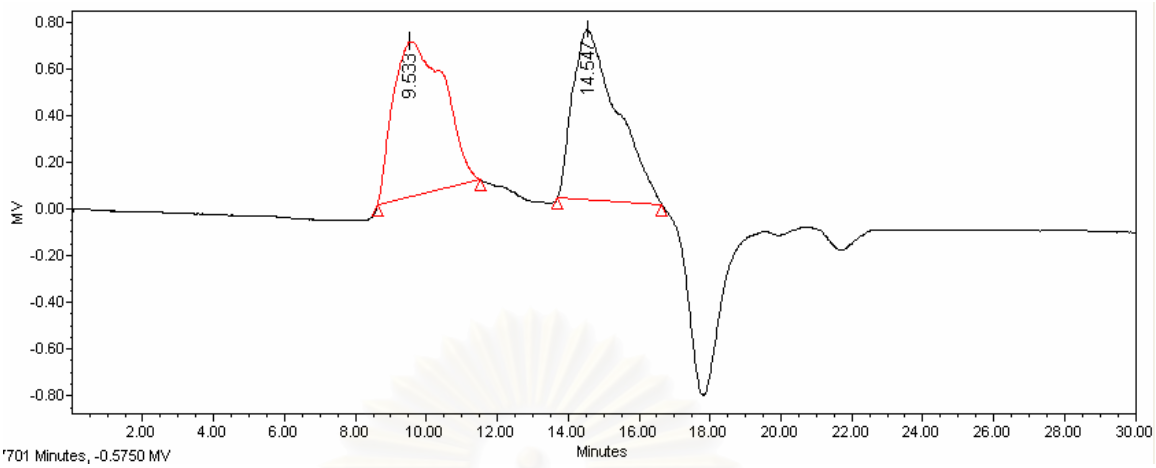


Fig 4.2 Size exclusion HPLC chromatogram of 1% crude xanthan gum produced from gelatinized cassava starch

From Fig 4.1, The HPLC chromatogram of commercial food grade xanthan gum showed only one peak (at 9.469 min) represented molecular weight of xanthan gum, more than 788,000 Da (pullulan was used as standard). However, the HPLC chromatogram of crude xanthan gum from our experiment showed 2 peaks (9.533 and 14.547 min) at the molecular weight higher than 788,000 Dalton and lower than 22,800 Dalton while the average molecular weight of xanthan gum were varied from 2 x 10^6 to 20 x 10^6 Dalton (Fig 4.2). The results indicated that the produced xanthan gum from gelatinized cassava starch had impurity.

Because of high impurity of xanthan gum produced from gelatinized cassava starch, the downstream purification steps for xanthan gum produced from gelatinized starch could be more complicate. From this reasons, we designed to use raw cassava starch instead of gelatinized form because the raw starch granule could not dissolve in the cultured medium (Whistler, BeMiller, and Paschall, 1984). The remaining unhydrolyzed starch could be separated by centrifugation and the impurity should be lower than using gelatinized starch as a carbon source. The size

exclusion HPLC chromatogram of xanthan gum produced from raw cassava starch was shown in Fig 4.3.

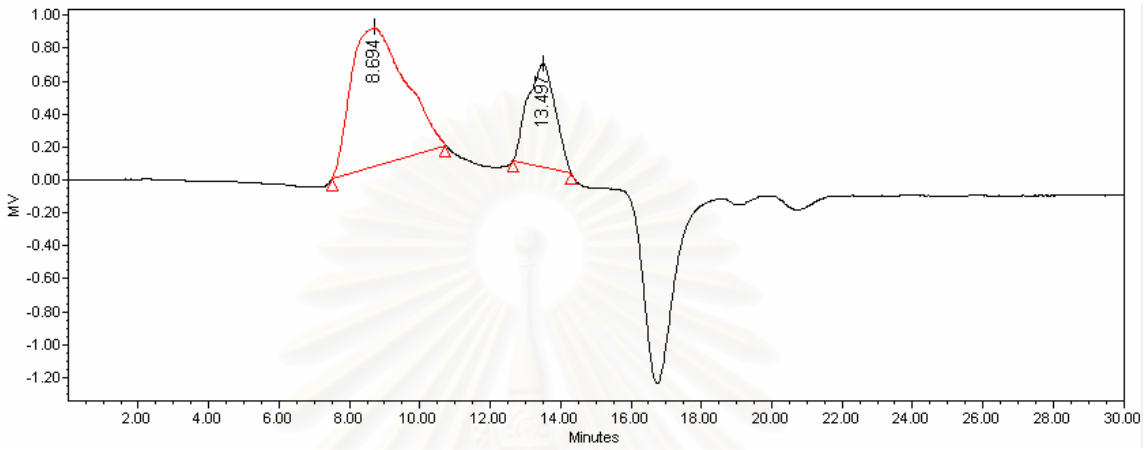


Fig 4.3 Size exclusion HPLC chromatogram of 1% crude xanthan gum produced from raw cassava starch

The results from Fig 4.3 showed that crude xanthan gum produced from raw cassava starch had low molecular weight impurity as same as crude xanthan gum produced from gelatinized cassava starch. However, percent peak area of high molecular weight component of crude xanthan gum produced from gelatinized starch was significantly lower than that found in the crude xanthan gum produced from raw starch (Table 4.2).

Table 4.2 Retention time and percentage of high molecular weight peak area of commercial grade and crude xanthan gum produced by cassava starch

Treatment	% peak area	Retention time (min)
Gelatinized starch	$51.03^{c} \pm 0.18$	$9.38^{a} \pm 0.21$
Raw starch	$74.05^{b} \pm 2.00$	$9.37^{a} \pm 0.20$
Commercial xanthan gum	$100.00^{a} \pm 0.00$	$9.38^{a} \pm 0.12$

$^{a,b,\cdots}$ is the significantly different ($p \leq 0.05$) at 3 replicates of each treatment

In addition, the high concentration of gelatinized starch could not be used in the industrial fermentation system because it has very high viscosity that effect on oxygen transfer in the system (Asim and Ghosh, 1999). Moreover, the gelatinization process requiring the addition of heat energy to starch granule slurries until the gelatinization temperature of the starch was exceeded. The whole process required a high-energy input (Shariffa *et al*., 2009). In order to solve these problems, we designed to use the medium containing raw cassava starch to reduce impurities because raw starch could precipitate and easy to separate by centrifugation and the high viscosity of the medium could be avoided.

However, the xanthan gum production using raw cassava starch as a sole carbon source was significantly lower than using gelatinized cassava starch (Table 4.2). To explain this result, the amylase production of *X. campestris* was checked to prove the ability to hydrolyze raw cassava starch. In the next experiment, we studied the ability to produce amylase of *X. campestris* in raw cassava starch medium.

4.2 Effect of raw cassava starch on amylase production by *X. campestris* TISTR 840

There were many researchers studied in amylase production of bacteria and mold, they found that most amylase was an inducible enzyme and was generally induced in the presence of starch or its hydrolytic products (Tonomura *et al.*, 1961; Morkeberg, Carlsen, and Neilsen, 1995). In the case of *X. campestris*, Tseng and Peng (1985) reported that this bacteria could produce amylase very well when cells were grown in the medium contain maltose or soluble starch.

In this study, raw cassava starch was used to induce amylase production of *X. campestris*. The sterilized raw cassava starch (1.0%) was mixed in 3 liters of sterilized XOL basal medium in a 5 liters reactor. The cells were pre-grown overnight in yeast malt extract (YM) broth then transfer 10% of the overnight cells to new fresh YM broth and incubated for 24 h. The culture were harvested and washed twice with sterilized water, these cells were used as starter.

X. campestris could grow and produced amylase when cultured in XOL medium using raw cassava starch as a sole carbon source, the result is showed in Fig 4.4 The highest activity of amylase was found on 16 h of cultivation at 0.277 ± 0.002 U/ml. It was very low as compared with amylase produced from *Bacillus* sp. The *Bacillus* sp. produced 15 to more than 100 U/ml of amylase depended on strains, cultured conditions, and cultured media composition (Bajpai, Gera, and Bajpai, 1992; Mamo and Gessesse, 1999; Tanyildizi, Özer, and Elibol, 2005; and Rajagopalan and Krishnan, 2008).

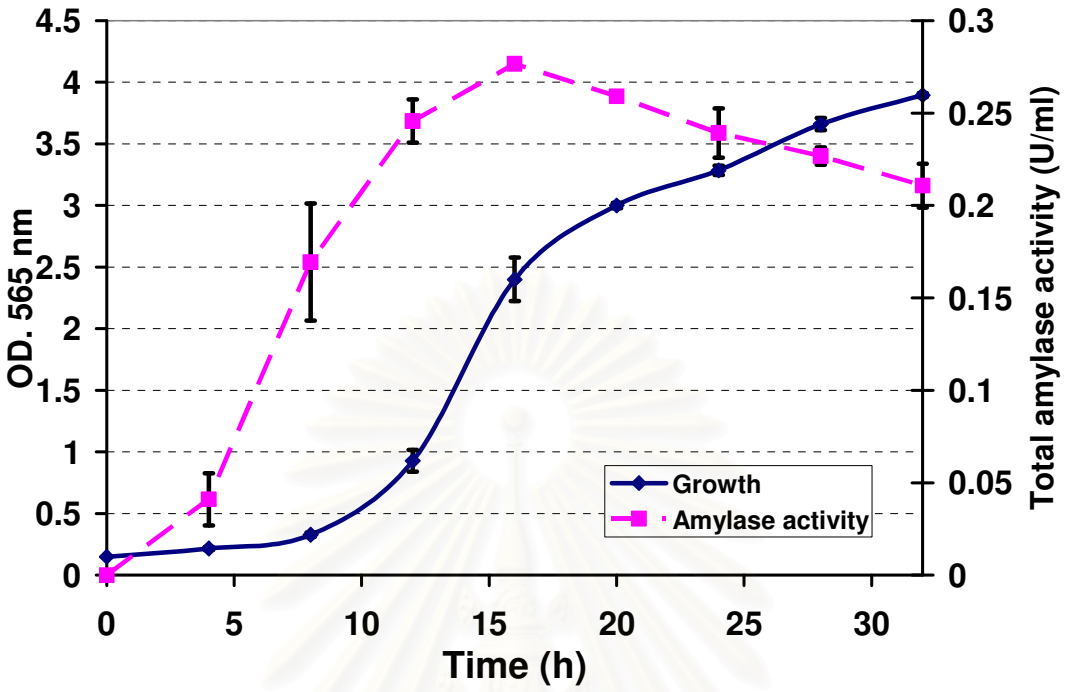


Fig 4.4 Amylase activity as a function of cell growth, The starter of *X. campestris* was inoculated in 3 liter of XOL medium containing 1.0% raw cassava starch as carbon source in a 5 liter bioreactor. The cells were incubated at 30°C, pH 7.0, aeration rate 0.5 vvm, agitation speed 200 rpm, for 32 h.

Moreover, amylase production of *X. campestris* using raw cassava starch as a carbon source was lower than using maltose and gelatinized starch when cultured in the bioreactor. The results show in Table 4.3. Because of the low ability of *X. campestris* to produced amylase when grown in raw cassava starch, therefore production of reducing sugar in the fermentation system was very low (Fig 4.5).

Table 4.3 Effect of different carbon sources on amylase production of *X. campestris*

Substrate	Total activity of amylase (U/ml)
glucose	$0.0031^{d} \pm 0.0002$
Maltose	$0.4108^{a} \pm 0.0019$
Gelatinized cassava starch	$0.3500^{b} \pm 0.0116$
Raw cassava starch	$0.2770^{c} \pm 0.0020$

$^{a,b\ldots}$ show the significantly different ($p \leq 0.05$) with 3 replications in each substrate

From Table 4.1, the results showed that *X. campestris* produced enzyme amylase when cultured in XOL medium containing glucose, maltose, gelatinized and raw cassava starch as a carbon source. Maltose was the best inducer for amylase production by *X. campestris*. This result confirmed by the report of Tseng and Peng (1985). They used 8 types of sugar and soluble starch as substrates for amylase production by *X. campestris* and found that maltose was the best inducer.

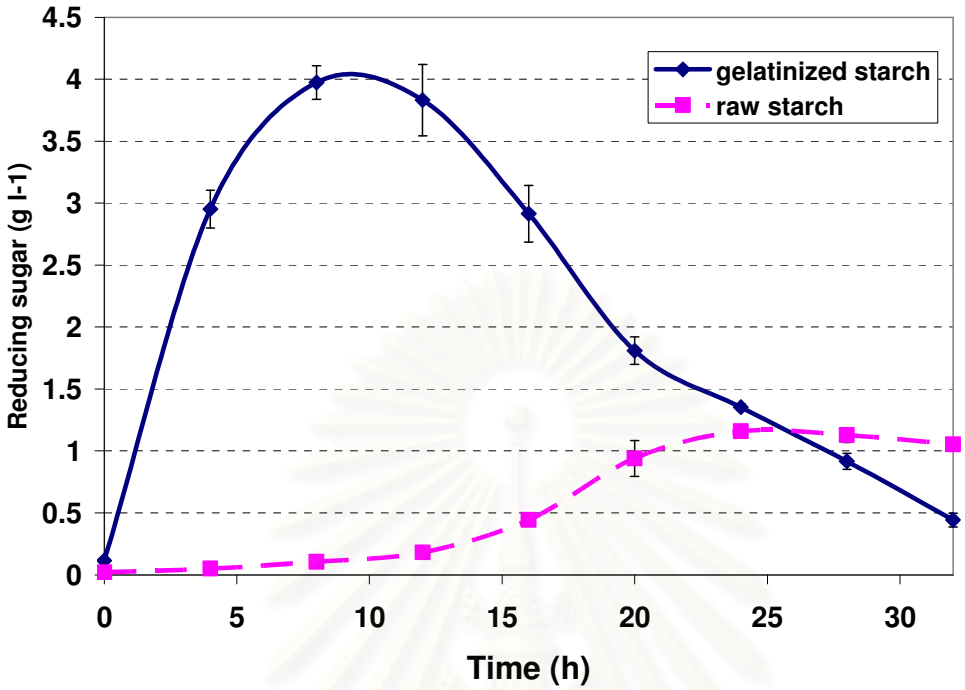


Fig 4.5 Reducing sugar content over time of fermentation of *X. campestris* cultured in 3 liters of XOL medium containing 1.0% gelatinized or raw cassava starch as a carbon source in a 5 liter bioreactor, incubated at 30°C, pH 7.0, aeration rate 0.5 vvm, agitation speed 200 rpm.

Low reducing sugar content influenced growth rate of *X. campestris* when raw cassava starch was used as carbon source. The slow growth of *X. campestris* affected the production of amylase. The same result found in amylase production by *Bacillus caldolyticus* and *Bacillus amyloliquefaciens* (Emanuilova and Toda, 1984; Hillier *et al.*, 1999). They found that growth rate of *Bacillus* involved in the amylase production mechanism. However, the amylase enzyme by *X. campestris* could hydrolyze raw cassava starch granules. To confirm this result, the scanning electron microscope of starch granule remained in bioreactor at the end of incubation was examined. The result is showed in Fig 4.6

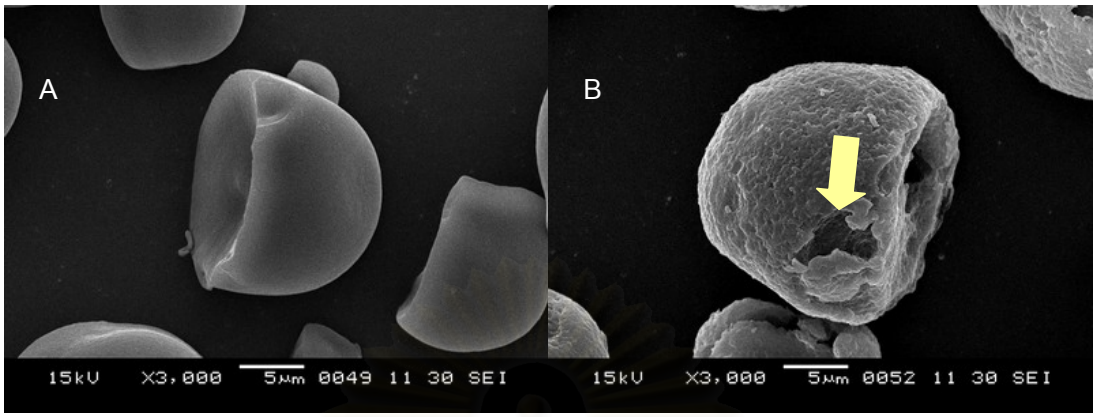


Fig 4.6 SEM chromatogram of raw cassava starch granules showing erosion of the granule surface after 120 h fermentation (B) comparing with unfermented granules (A)

The scanning electron microscopy of the raw cassava starch after 120 h of cultivation indicated that the granules were damaged. Fig 4.6-A showed the surfaces of native cassava starch granules were smooth, whereas Fig 4.6-B showed the damaged surfaces of digested raw cassava starch granules. The arrows showed the starch granules were randomly distributed due to the breakdown of the granules by the enzyme. This supported the efficient action of amylase produced by *X. campestris* toward raw cassava starch granules.

There were many researchers reported about action of amylase produced by some bacteria on raw starch granules. Goyal, Gupta, and Soni (2005) reported the direct hydrolysis of raw potato starch by thermostable α-amylase from *Bacillus* sp. From the scanning electron micrograph, they found the damage of starch granules on the surface and that granules were not completely damaged. The same result of amylase action from *Lactobacillus plantarum* was reported by Giraud, Champailler, and Raimbault (1994). They found that the raw starch granules had a crystalline

appearance similar to the native granules, but the hydrolyzed granules surface was rougher than that native. The α-amylase has limited activity on raw starch granules because granules are very resistant to amylolytic digestion (Hyun and Zekius, 1985). The mechanism of adsorption of amylase on starch granules was still unclear, but binding probably occurs through a C-terminal binding domain (Demirkan *et al.*, 2005)

Although the ability to produced amylase to hydrolyzed raw cassava starch of *X. campestris* was very low but this microorganism could grow and produced xanthan gum gum in medium contained raw cassava starch (Table 4.1). The xanthan gum yields were lower than production by using 4% glucose as substrate that could produce 30 g/l of xanthan gum in 96 h of fermentation (Weiss and Ollis, 1980). However, it was a little lower than xanthan gum production in other unmodified starch, soluble starch and potato starch, that showed 12.10 and 9.754 g/l of xanthan gum, respectively (Leela and Sharma, 2000), when gelatinized cassava starch was used.

Amylase production by *X. campestris* cultured in raw cassava starch was very low compared with the production by bacteria and mold that used in the industrial amylase production. Increasing the ability to produce the enzyme of *X. campestris* was a good way to increase reducing sugar in the fermentation system. However, factors that used to increase amylase production should be also useful for xanthan gum production. The factors usually used for increasing amylase production were carbon and nitrogen source concentration, oxygen transfer rate, temperature, pH, and ions used in cultured medium (Milner, Martin, and Smith, 1996; Dey et al., 2001; Tanyildizi, Özer, and Elibol, 2005). Likewise, the factors for optimized xanthan gum

production were carbon and nitrogen ratio, oxygen transfer rate, temperature, pH and mineral composition in the medium (Garcia-Ochoa *et al*., 2000; Lin and Chen, 2007). Because of temperature, pH, and ions affected on amylase and xanthan gum production and these factors also affected on amylase activity. In the next experiment, effect of temperature, pH, and ions used in the medium on amylase activity were studied to confirm that the optimum condition for xanthan gum and amylase production were the same.

4.3 Partial purification and characterization of amylase produce by *X. campestris* TISTR 840

Since there were many factors affected on amylase and xanthan gum production by *X. campestris*, effect of growth condition and composition of the medium were also important to amylase activity. The culture conditions such as temperature and pH, and ions from ingredients in the medium might interrupt the action of amylase. Therefore, the effects of those factors on amylase activity will provide important information for xanthan gum production using raw cassava starch as a sole carbon source.

4.3.1 Partial purification of amylase produced by X. campestris TISTR 840

The amylase was partial purified through centrifugation, microfiltration and ultrafiltration. Culture medium contained raw cassava starch was collected at 16 h then centrifuged to separate cells and the remaining starch granules. The supernatant was filtered through a microfiltration membrane, pour size 0.45 μm, to separate xanthan gum that has molecular weight more than 100,000 Daltons. Then the solution was filtered through the 0.10 μm ultrafiltration membrane to concentrate enzyme (Tseng and Peng 1985). The micro molecules, such as mineral, were removed by dialysis against cold water at 4°C and the partial purified amylase was lyophilized and kept at -20°C. The results of partial purification were shown in Table 4.4.

Table 4.4 Partial purification of amylase produced by *X. campestris* TISTR 840.

Step	Volume (ml)	Total activity (U/ml)	Specific activity (U/mg)	Purification fold
Crude enzyme	2000	0.232	0.224	1
Microfiltration	1450	0.249	0.251	1.120
Ultrafiltration	200	0.310	0.963	4.299
Freeze-drying	30	0.514	2.089	9.326

4.3.2 Effect of temperature on partial purified amylase

For estimation of the optimum temperature of the enzyme, the activity was determined at different temperature, 30 – 75°C. The highest determining activity in the experiment was set to 100% relative activity. The enzyme showed high activity from above 30°C and reached the peak at 40°C. However, the activity declined sharply at temperatures higher than 45°C (Fig 4.7), and could not be detectable at 75°C. The same results reported by Tseng and Peng (1985), who purified α-amylase from *Xanthomonas campestris* strain 11 and found that the activity reach to peak at 45°C but the activity was gone after 5 minutes pre-incubation period at 55°C.

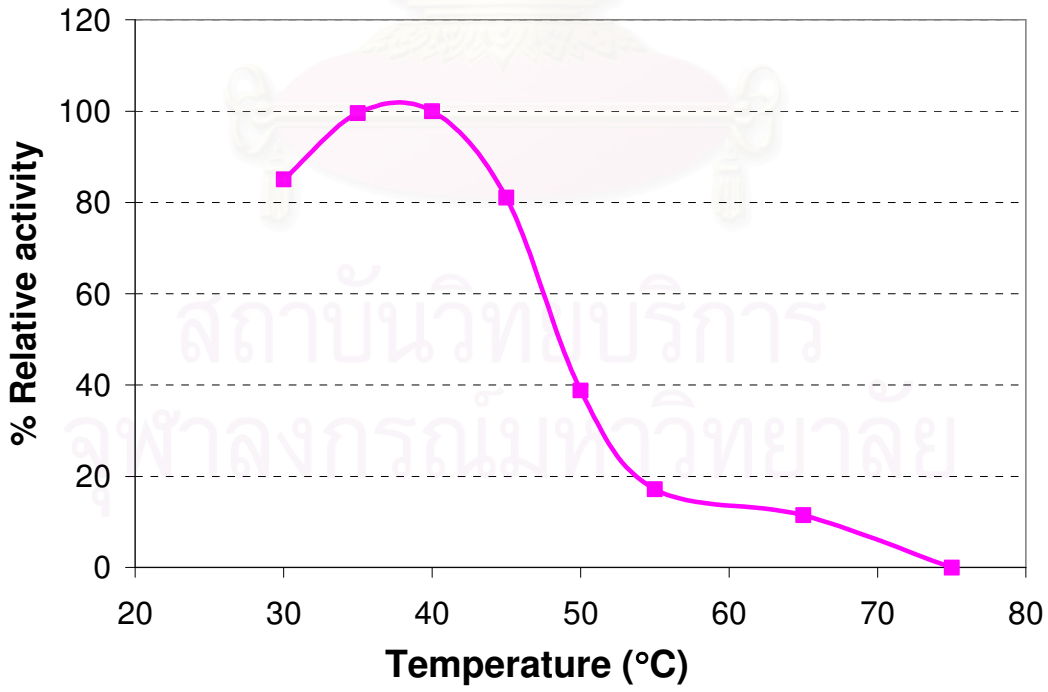


Fig 4.7 Effect of assay temperature on partial purified amylase activity, the percentage of relative activity was mean of 3 replication

The highest amylase activity was found at 40°C which was the temperature that *X. campestris* could not growth (Lilly, Wilson, and Leach, 1985). However, the activity at this temperature was 14.98 % higher than the activity found at 30°C, the optimum temperature for xanthan gum production.

4.3.3 Effect of pH on partial purified amylase

In order to observe the effect of pH on amylase activity, the enzyme was assayed at different pH values by using McIlvaine (Appendix A) Universal buffer to cover the alkaline, neutral, and acidic area. The enzyme exhibited the highest activity at neutral pH 7.0 (100% relative activity), it was 0.478 U/ml, result shows in Fig 4.8. It was also the optimum pH for xanthan gum production (Kang and Cottrell, 1979). Tseng and Peng (1985) reported that the α-amylase from *X. campestris* pv *campestris* exhibited the highest activities at neutral pH, 6.0 – 7.0. However, the optimum pH of α-amylase produced by *Bacillus* sp. seem to be shift to the alkaline area, pH 8 – 10 (Burhan *et al*., 2003) and shifted to the acid area, pH 5 – 6, in case of *Clostridium acetobutylicum* amylase (Paquet *et al*., 1990).

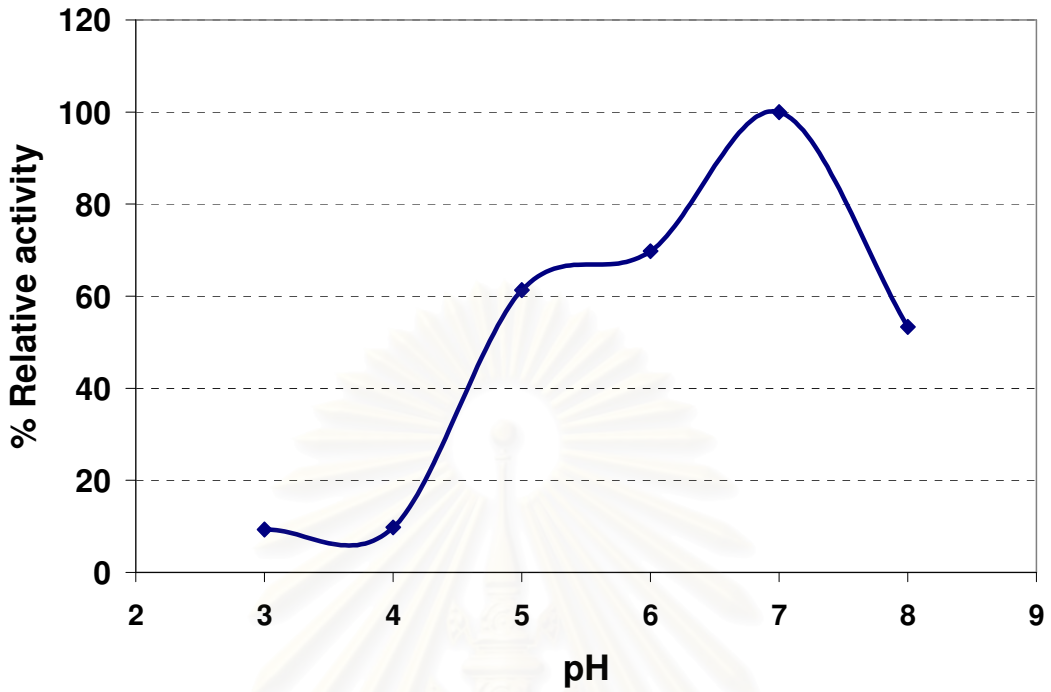


Fig 4.8 Effect of pH on partial purified amylase produced by *X. campestris*, the percentage of relative activity was mean of 3 replications

4.3.4 Effect of ions on amylase activity

There were many researchers reported the effect of ions on amylase activity produced by bacteria, particularly *Bacillus* sp (Demirkan *et al.*, 2005). To observe effect of ions on amylase activity produced by *X. campestris*, $FeSO_4$, $MgCl_2$, $CaCl_2$, and $MnCl_2$ at 5.0 mM of the final concentration was included in the reaction mixture. Ions used in this assay came from the composition of ions included in XOL and the production medium (Thanyaporn, 1999) that always used in xanthan gum production. The results show in Table 4.4.

Table 4.5 Effect of ions on amylase activity

Ion	Relative activity* (%)
Control	100
Fe^{2+}	69.71
Mg^{2+}	95.49
Ca^{2+}	102.24
Mn^{2+}	97.58

*The results were mean of 3 replications

Table 4.5 shows effect of some ions on amylase produced by *X. campestris* TISTR 840. The ferric ion was found to inhibit amylase activity, while the rest of the ions caused little effect on the enzyme activity. The same result was reported by Tseng and Peng (1985), they found that Cu^{2+} and Fe^{2+} were 35% and 12% inhibited α-amylase produced by *X. campestris* pv *campestris*. Moreover, it was also reported that Fe^{2+} can inhibit α-amylase produced from *Bacillus amyloliquefaciens* (Demirkan *et al.*, 2005) and *Aspergillus carbonarious* (Okolo *et al*., 2000).

From the characterized experiments, it was known that cultured temperature and pH were the good conditions for amylase action as same as for growth and xanthan gum production of *X. campestris*. Although, Mg^{2+}, Mn^{2+}, and Ca^{2+} in cultured medium had no effect on amylase but Fe^{2+} could inhibit amylase. XOL and Roseiro media might have effect on amylase produced from *X. campestris* because the media had $FeCl_2$ and $FeSO_4$ as their composition, respectively. However, concentration of Fe^{2+} in XOL media were 0.0042 – 0.01 g/l, they were lower than

concentration of Fe^{2+} used in this experiment. The Fe^{2+} had a small effect on amylase activity because concentration of ion also affected on activity of enzyme (Onishi, 1972).

The results of experiment 4.1 – 4.3 showed the possibility to use cassava starch as carbon source. The fermentation condition and ion composition in culture medium were suitable for *X. campestris*. From the information described above, it indicated that C:N ratio and aeration rate were the other factors that should increase amylase production. This results tent to lead to increasing of reducing sugar in the fermentation system and xanthan gum production should be increase. However, XOL minimal medium used in this experiment contained 0.125% peptone to support growth of the bacterium. Since peptone is known as a complex ingredient that contain both of carbon and nitrogen, this ingredient is the good supplement for production of amylase in bacteria and fungus such as *Bacillus* sp. and *Aspergillus* sp. (Kundu, Das, and Gupta, 1973; Tanyildizi, Özer and Elibol, 2005; Hernández *et al.*, 2005). From this reason, it is important to study the effect of peptone on growth and amylase production of *X. campestris* to avoid interference of peptone in the future studies.

4.4 **Effect of peptone on growth and amylase production of *X. campestris* TISTR 840**

From the preliminary experiment, *X. campestris* was cultured in the medium containing raw cassava starch without peptone and the results showed very slow growth rate. Gelatinized cassava starch was then used as carbon source instead of raw starch to observe effect of peptone on growth and amylase production of *X. campestris*. The result shows in Fig 4.9 to 4.11.

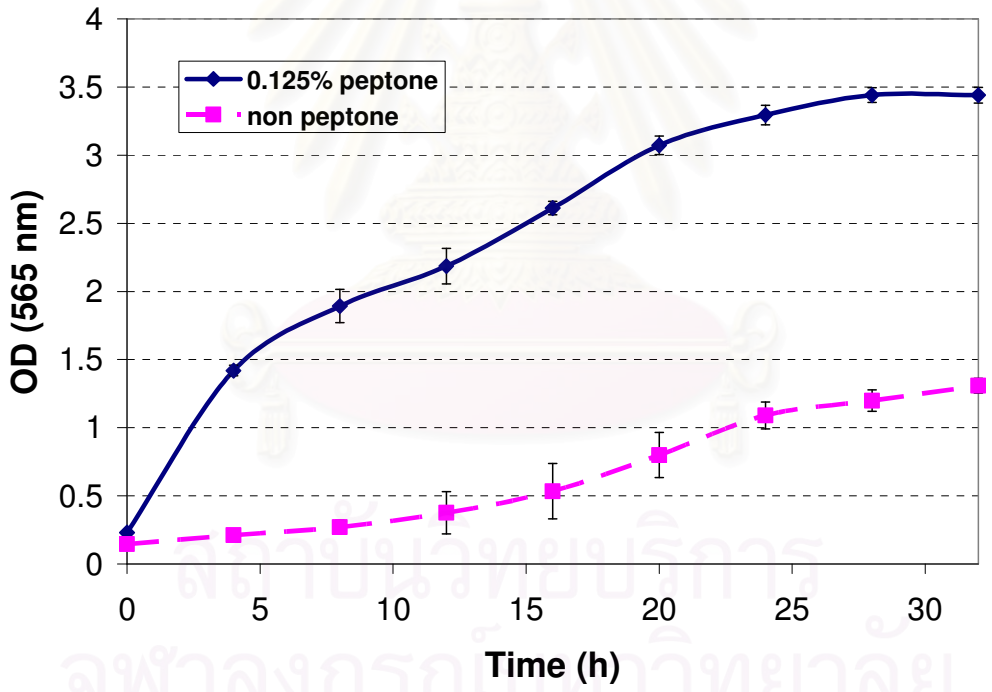


Fig 4.9 Growth curve of *X. campestris* cultured in 3 liters of XOL medium with 0.125% and without peptone using 1.0% gelatinized cassava starch as carbon source in a 5 liters bioreactor. The cells were incubated at 30°C, pH 7.0, aeration rate 0.5 vvm, agitation speed 200 rpm, for 32 h

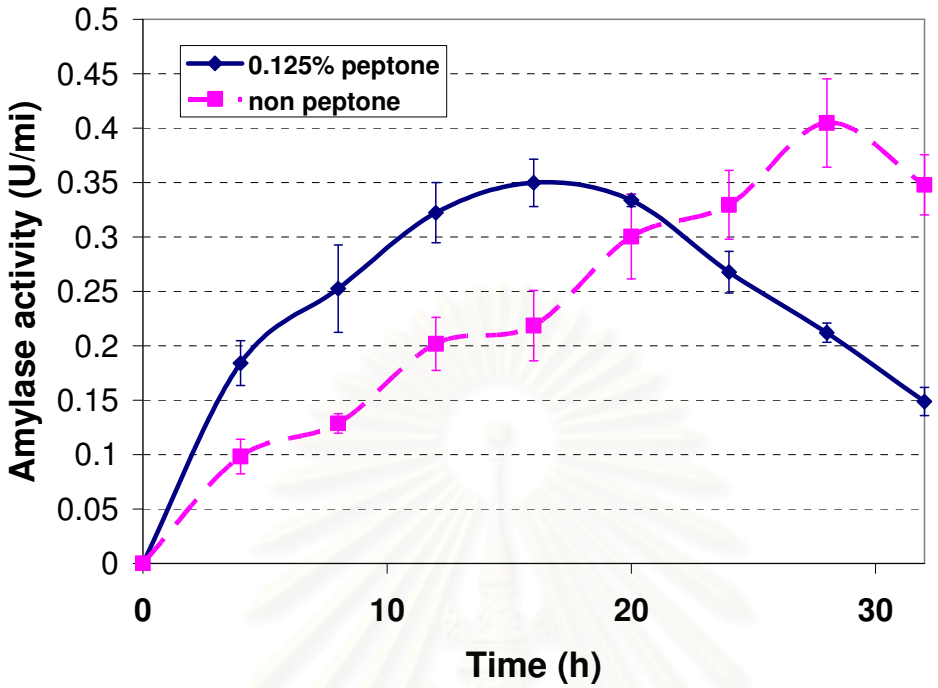


Fig 4.10 Amylase activity over time of cultivation of *X. campestris* cultured in 3 liters of XOL medium with 0.125% and without peptone using 1.0% gelatinized cassava starch as carbon source in a 5 liter bioreactor. The cells were incubated at 30°C, pH 7.0, aeration rate 0.5 vvm, agitation speed 200 rpm, for 32 h

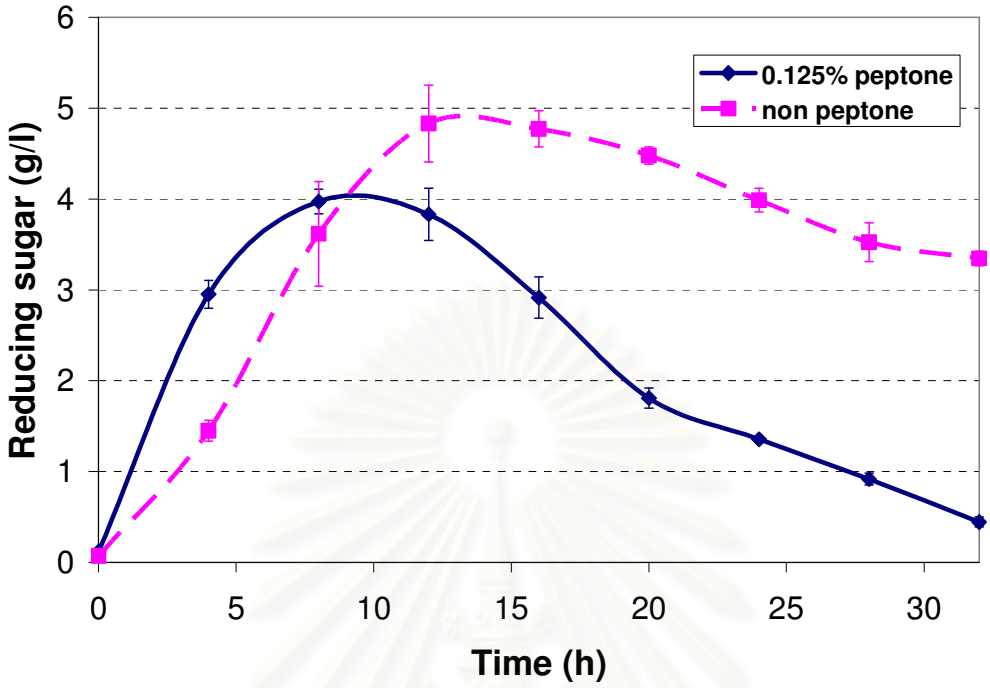


Fig 4.11 Reducing sugar over time of cultivation of *X. campestris* cultured in 3 liters of XOL medium with 0.125% and without peptone using 1.0% gelatinized cassava starch as carbon source in a 5 liter bioreactor. The cells were incubated at 30°C, pH 7.0, aeration rate 0.5 vvm, agitation speed 200 rpm, for 32 h

Tryptone or peptone have been reported to support growth and amylase production of *Bacillus lichenniformis* (Chandra, Medda, and Bhadra, 1980) and *Bacillus thermooleovorans* NP54 (Malhotra, Noorwez, and Satyanarayana, 2000). Fig 4.9 showed that, using non peptone XOL medium significantly affected on growth of *X. campestris*. Although, amylase activity could detect and increase continuously in the system that had no peptone (Fig 10) and reducing sugar after 12 h of cultivation was significantly higher than in the system contain peptone (Fig 4.11). However, very slow growth rate indicated slow metabolism of *X. campestris*. Thomas, Hynes, and Ingledew (1996) reported that the chemical composition of the

culture medium, particularly the nitrogen source, is a mean of physiological control and regulation of microorganism metabolism and type of nitrogen source effect on bacteria growth. Because of the slow metabolism of *X. campestris*, the reducing sugar in the fermentation system without peptone was decreased slower than in the system containing peptone. Moreover, in this bioreactor, the pH was controlled at 7.0 ± 0.1 in the entire batch (Fig 4.12). The most of reducing sugar in this system came from the hydrolysis of amylase and not included with acid hydrolysis. The highest amylase productions in both XOL media, with 0.125% and without peptone, were not significant different (Fig 4.10). However, growth rate of *X. campestris* stilled very low when grown in the medium without peptone. It suggested that enzyme secretion was not growth associate (Shinmyo, Kimura, and Okada, 1982; Roychoudhary, Parulekar, and Weigand, 1989; and Malhotra, Noorwez, and Satyanarayana , 2000.

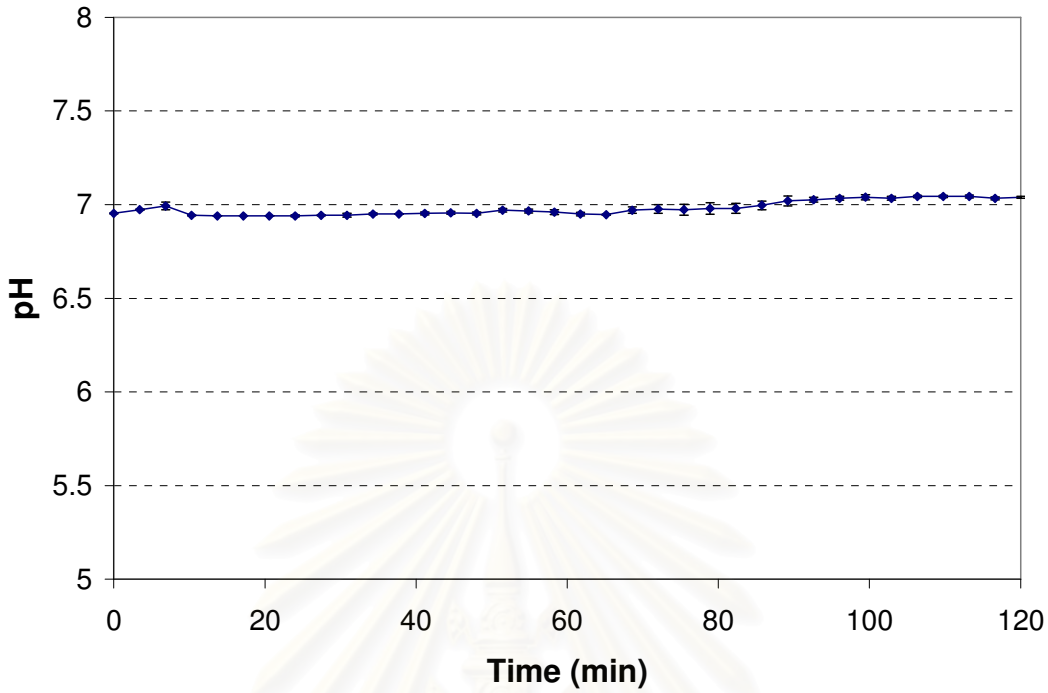


Fig 4.12 The pH profile of the fermentation system by *X. campestris* cultured in XOL medium without peptone.

The results indicated that peptone was necessary for growth of *X. campestris* when cultured in cassava starch and good growth of this bacterium required for xanthan gum production (Garcia-Ochoa, Garcia-leon, and Romero, 1990). From this reason, carbon and nitrogen content in peptone and cassava starch were detected by CHNS/O analyzer to calculate real C:N ratio for the next experiment. The results show in Table 4.6.

Table 4.6 Percentage of carbon and nitrogen in cassava starch and peptone used in XOL medium

Sample	Percentage of Carbon* (%C)	Percentage of Nitrogen* (%N)
Cassava starch	39.675	0.0
Peptone	41.844	12.156

*The results were mean of 3 replications

Because of the C:N ratio and aeration were the important factors for optimization of both amylase and xanthan gum production. Effect of C:N ratio and aeration rate on amylase production of *X. campestris* was studied.

4.5 Effect of C:N ratio and aeration rate on amylase and xanthan gum production

In this experiment, raw cassava starch and ammonium sulfate were used as carbon and nitrogen source. The concentration of ammonium sulfate was fixed at 0.1% because high concentration of ammonium is toxic to some bacteria. It has been found to be detrimental to enzyme production causing, in some cases, a complete inhibition of amylase production (Kole and Gerson, 1989). To study the effect of C:N ratio, the nitrogen concentration of ammonium sulfate and peptone was controlled.

Aeration rate was varied as 0.0, 0.5, and 1.0 vvm because when the aeration rate was higher than 1.0 vvm, it was very hard to control level of foam in fermentation

system. High level of foam after 48 h of cultivation was also an important problem to increase the risk of contamination. In case of C:N ratio, we had the previous information of various C:N ratio form 10:1 to 70:1 to observe effect of raw cassava starch on xanthan gum production. The results show in Fig 4.13.

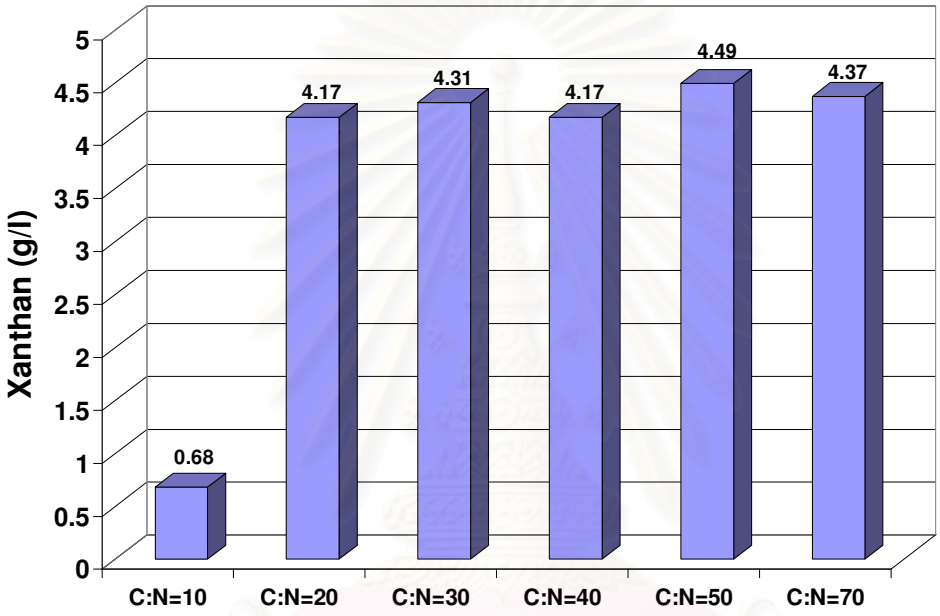


Fig 4.13 Xanthan gum yield produce by *X. campestris* cultured in different C:N ratio. Cells were cultured in 3 liter of XOL medium contained raw cassava starch in a 5 liter bioreactor. The cells were incubated at 30°C, pH 7.0, agitation speed 200 rpm, for 120 h the xanthan yields were mean of 2 replications

From Fig 4.13, the xanthan gum content was stable at 4.17 – 4.49 g/l when C:N ratio was change from 20:1 to 70:1. Furthermore, increasing the aeration rate showed significant effect on xanthan gum production. To explain effect of C:N ratio and aeration rate, the central composite design was used. The C:N ratio and aeration

rate were varied form 10:1 to 30:1 and 0.0 to 1.0 vvm, respectively. The possibility to predict effect of both factors on amylase and xanthan gum production was studied and the relationship between C:N ratio and aeration rate was also observed.

4.5.1 Model fitting and response surface plotting

The levels of independent variables in coded and uncoded form according to the experimental design and response (highest amylase activity, γ_1, and xanthan gum yield, γ_2) were shown in Table 4.6. Each row represents one experiment. The experiments were randomized in order to maximize the effects of unexplained variability in the observed responses due to extraneous factors.

The regression coefficient equation of amylase production and the amount of xanthan gum are presented in Table 4.7. The significant terms were used in equations that could predict the responses, γ_1 and γ_2, because they effected on the responses at $p \leq 0.05$. From Table 4.8, the coefficient of aeration rate is higher than the coefficient of C:N ratio. It indicated that aeration had more effect on highest amylase activity and xanthan gum yield than C:N ratio.

Table 4.7 Central composite design (conditions and responses) for amylase and xanthan gum production

Run	Conditions		Responses	
	C:N ratio x_1	**Aeration rate (vvm), x_2**	**Highest amylase activity (U/ml), γ_1**	**Xanthan gum yield (g/l), γ_2**
1	10 (-1)	0.0 (-1)	0.195	0.730
2	30 (+1)	0.0 (-1)	0.192	0.897
3	10 (-1)	1.0 (+1)	0.258	3.340
4	30 (+1)	1.0 (+1)	0.344	7.947
5	10 (-1)	0.5 (0)	0.244	1.643
6	30 (+1)	0.5 (0)	0.242	4.303
7	20 (0)	0.0 (-1)	0.187	0.810
8	20 (0)	1.0 (+1)	0.278	6.310
9	20 (0)	0.5 (0)	0.251	4.173
10	20 (0)	0.5 (0)	0.253	4.213
11	20 (0)	0.5 (0)	0.248	4.110
12	20 (0)	0.5 (0)	0.256	4.140

Table 4.8 Regression coefficient (R^2) for highest amylase activity and xanthan gum yield produced by *X. campestris*

Coefficient	Highest amylase activity	Xanthan gum yield
β_0	0.249*	4.018*
β_1	0.011*	1.239*
β_2	0.046*	2.527*
$\beta_1\beta_2$	0.018*	1.110*
β^2_1	-0.004	-0.739*
β^2_2	9.649×10^{-5}	-0.153
R^2	0.895	0.986

Note: Subscripts; β_0 = Constant, β_1 = C:N ratio (x_1), β_2 = Aeration rate (x_2)
*Significant at 0.05 level

The resulted showed the coefficients of multiple determinations (R^2) were more than 0.85 ($p \leq 0.05$) in every parameter. Since the R^2 value give the percentage variation in the response explained by our regression, in case of highest amylase and xanthan gum production, we could explain 89.5% and 98.6% of the variation, respectively (Psomas, Liakopoulou-Kyriakides, and Kyriadis, 2007). Results indicated that these equations had a model fit with experimental data (Rastogi and Rashmi, 1999) which may be used to estimate xanthan gum production value after varying C:N ratio (x_1) and aeration rate (x_2). The relationship of highest amylase activity and xanthan gum yield can be predicted using equation 1 and 2 respectively.

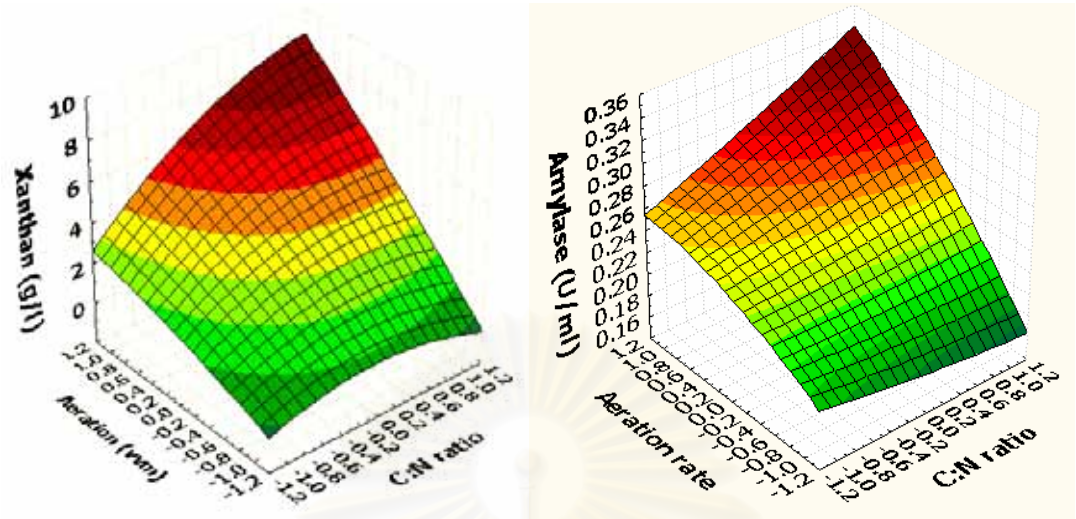
Highest amylase activity $= 0.249 + 0.011x_1 + 0.046x_2 + 0.018\ x_1x_2$

$(R^2 = 0.895, p \leq 0.05)$……..……Equation 1

Xanthan gum yield $= 4.018 + 1.239x_1 + 2.527x_2 - 0.739x_1^2 + 1.110x_1x_2$

$(R^2 = 0.986, p \leq 0.05)$……..…...Equation 2

From equation 1, the relationship between C:N ratio and aeration rate was shown in terms of linear and interaction and equation 2 was shown in terms of linear, quadratic and interaction. Increase in highest amylase activity and xanthan gum yield corresponded to a rise in the C:N ratio and aeration rate until a critical point was reached, at which point both factors were constant. It could be explain that more concentration of raw cassava starch gave high substrate concentration for amylase action. Since the rate of starch hydrolysis increased when substrate increase (Anprung, 2004), small molecule of reducing sugar (product of starch hydrolysis) was also increased and it presented high level of the simple sugar for *X. campestris* activities, including xanthan gum production. Furthermore, increasing of substrate, or carbon source, was significantly affected on xanthan gum production when the reducing sugar concentration in fermentation system was not more than 5% (Wemau, 1979).



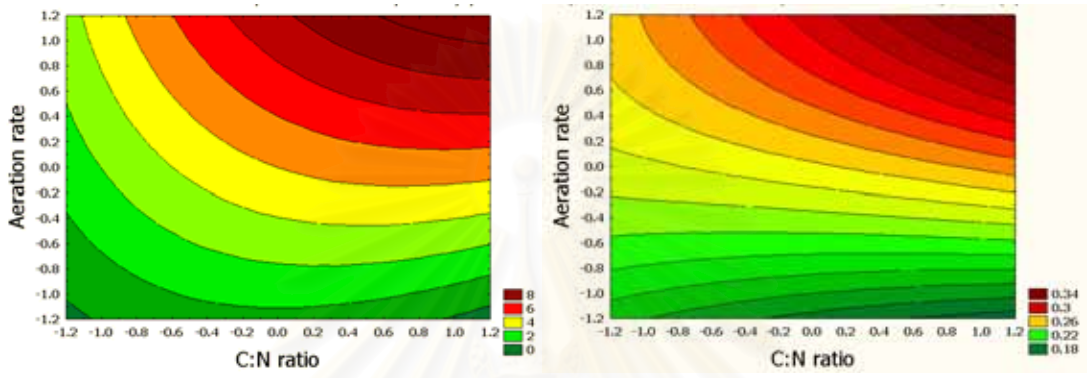
(A) **(B)**

Fig 4.14 Response surface showing effects of C:N ratio and aeration rate on xanthan gum yield (A) and amylase production (B).

According to Fig 4.14, which represents the relationship between C:N ratio (10:1 to 30:1) and aeration rate (0.0-1.0 vvm) on amylase production and xanthan gum yield. The increasing of C:N ratio involved in the increasing of aeration rate leads to increase the highest amylase activity and xanthan gum yield. However, aeration rate was the factor that had stronger effect than C:N ratio. Results form Table 4.6 indicated that the increasing of aeration rate enhanced amylase production in every C:N ratio but when C:N ratio increased at constant aeration rate, amylase activity was not always increased. The same result represented in the xanthan gum production. Moreover, there was a positive synergistic effect between C:N ratio and aeration rate on growth, amylase activity, and xanthan gum yield at $p \leq 0.05$.

4.5.2 Optimization

The contour plots of xanthan gum yield and amylase production of *X. campestris* were shown in Fig 4.15.



(A) **(B)**

Fig 4.15 Contour plots for optimization of xanthan gum (A) and amylase (B) production by *X. campestris*

From Fig 4.15, we found that the highest amylase production and xanthan gum yield were the same area. The optimal condition for amylase and xanthan gum production by *X. campestris* was C:N ratio as 27:1 to 30:1 (raw cassava starch and ammonium sulfate were used as carbon and nitrogen source, respectively) and using aeration rate at 0.88 – 1.0 vvm (Fig 4.16). Furthermore, the ranges that showed changing levels of amylase production were nearly the same area of the xanthan gum yield levels. It indicated that if we adjusted C:N ratio and aeration rate together and it affected on amylase production, and it was also affected on xanthan gum yield.

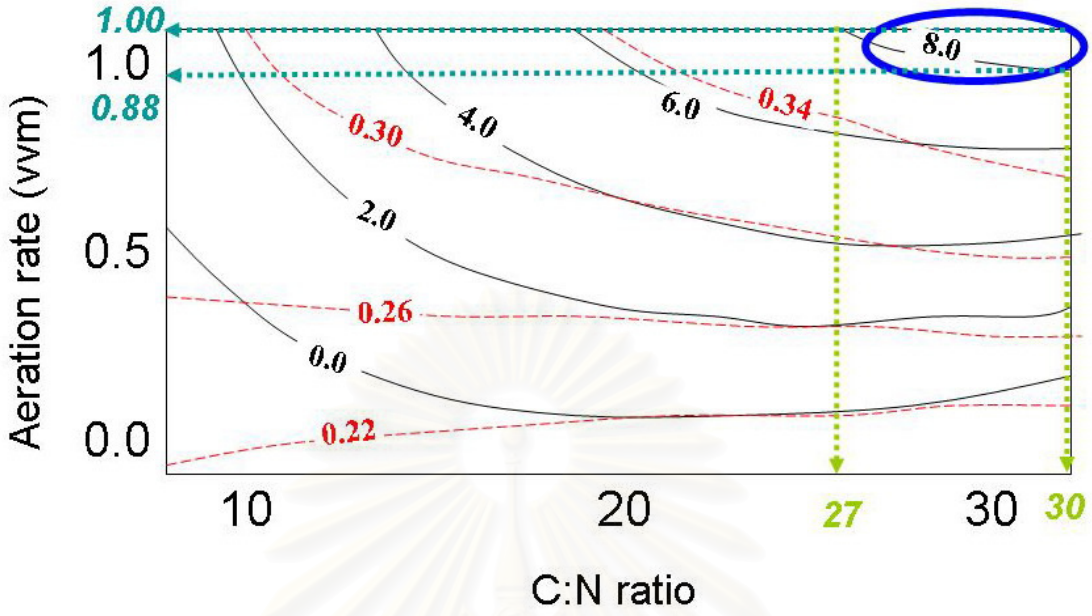


Fig 4.16 Superimposed contour plots for optimization of the highest amylase activity and xanthan gum yield

However, improving the composition of cultured medium and the cultured condition had little effect on amylase and xanthan gum production by *X. campestris* especially increasing of C:N ratio. Moreover, increasing aeration rate could increase 45.72% of xanthan gum yield when using C:N ratio as 30:1 but controlling level of foam in the bioreactor was a major problem. The other way to improve amylase production was mutation. Ethyl methansulfonate was used to mutate *X. campestris* and xanthan gum yield of the overproduced amylase strain was observed in low aeration rate fermentation system.

4.6 **Overproduction of amylase in *X. campestris* TISTR 840 and its effect on xanthan gum production in raw cassava starch medium**

4.6.1 Overproduction of amylase in X. campestris TISTR 840 by ethyl methanesulfonate (EMS)

X. campestris was treated with EMS and the survival curve was plot to determine the suitable time for EMS mutation. The survival curve was shown in Fig 4.17, That the survival rate of *X. campestris* was dropped rapidly in 0 to 20 h. Cells were reduced from 745,000 cells/ml at the initial to 98,200 cells/ml in 20 minute, which was 13.18% remaining of survival. The amount of cells remained closed to 10% at 30 minute treatment.

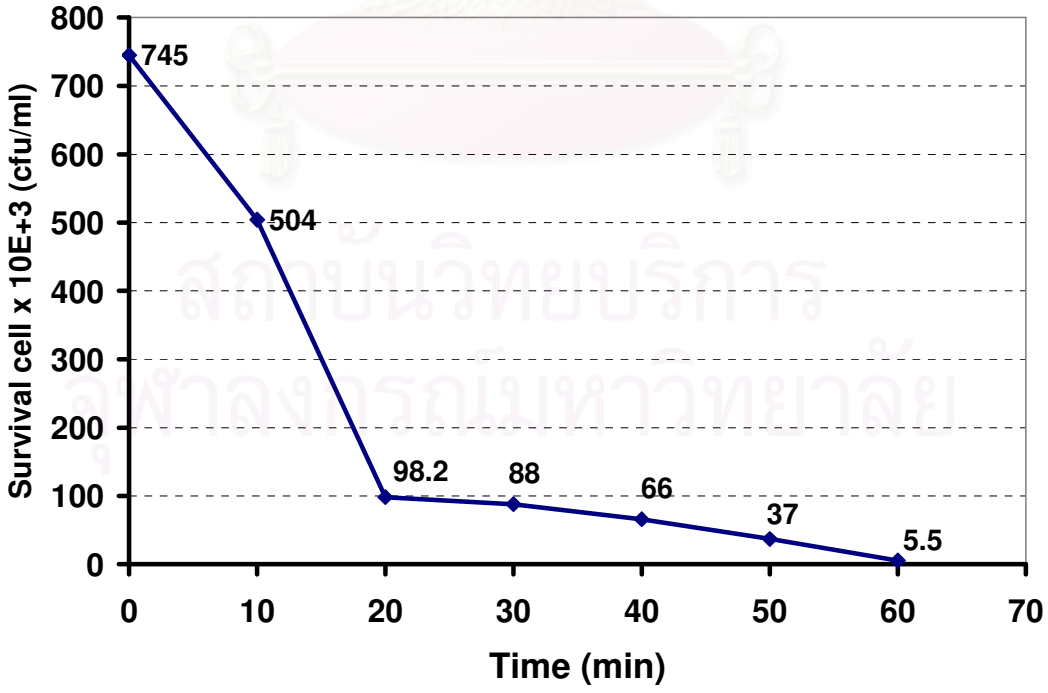


Fig 4.17 Survival curve of *X. campestris* treated with EMS

X. campestris was treated with EMS for 30 minutes, the survived cells was selected and study for the ability to produce amylase on starch agar plate. The colonies showed large clear zones in starch agar plates (Fig 4.18) were collected and they were cultured in XOL medium plus 1% raw cassava starch to test production of amylase and xanthan gum, in 250 ml flask contained 100 ml XOL medium using raw cassava starch as carbon source, initial pH was 7.0. They were incubated at room temperature and shaken at 200 rpm for 120 h. Samples were collected at 16 h and 120 h to measure amylase activity and xanthan gum content.

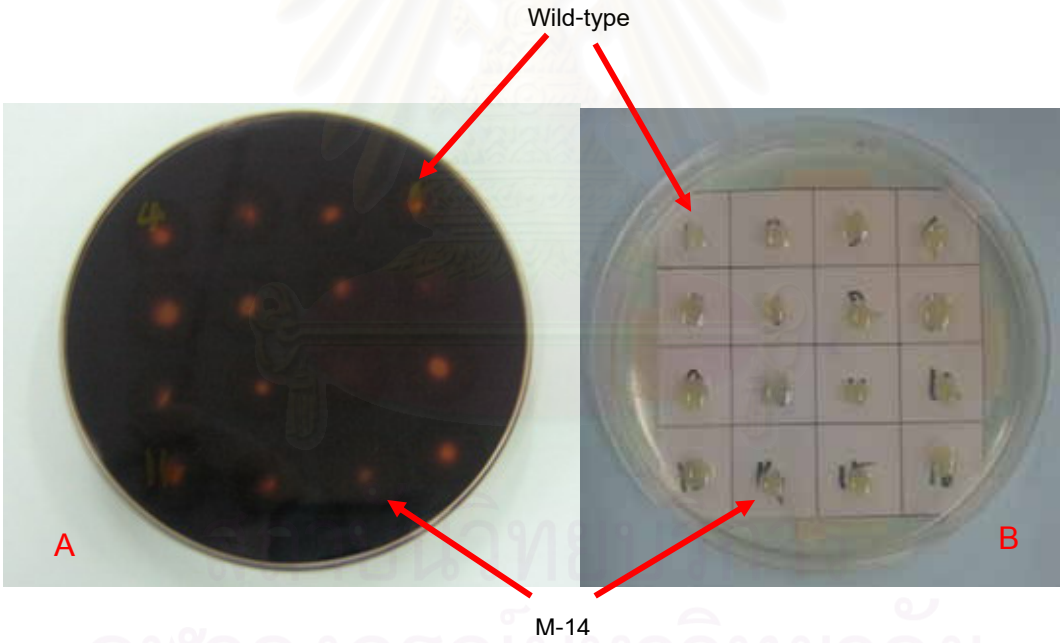


Fig 4.18 Colony morphology of *X. campestris* after treat with EMS for 30 minutes compare with wild-type, before dye (B) and after dye (A) with iodine solution to check the halo size around colonies

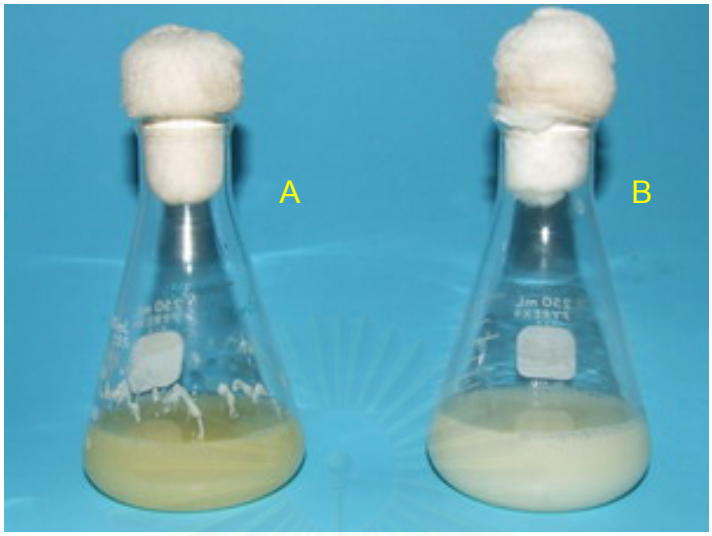


Fig 4.19 Cultured medium of mutant M-14 (A) and wild-type (B) of *X. campestris* after 120 h of cultivation

After treated with EMS, more than 400 colonies were tested for ability to produce amylase production ability on starch agar plate and several mutants of *X. campestris* that could overproduce amylase were obtained. The mutant could produce highest amylase activity was designated as M-14. No difference was observed between M-14 and wild-type in colony morphology (Fig 4.18-B). Amylase activity of mutant strain in both of XOL minimal and production medium were higher than wild-type strain (Table 4.9). Xanthan gum production of mutant in production medium (Thanyaporn, 1999) was 7.02 g/l, 2.36-folds higher than wild-type, 3.12 g/l.

Table 4.9 Amylase activity and xanthan gum production of M-14 and *X. campestris* wild-type cultured in 250 ml flask

Strain	Amylase activity* (U/ml)		Xanthan gum* (g/l)	
	XOL medium	Production medium	XOL medium	Production medium
M-14	0.045	0.063	2.40	7.22
Wild-type	0.034	0.057	1.86	3.12

* The results were mean of 3 replications

4.6.2 Effect of overproduction of amylase production in X. campestris TISTR 840 on xanthan gum production

The mutant strain was cultured in 5 liters bioreactor contained 3 liters of XOL medium plus 0.125% peptone, raw cassava starch and ammonium sulfate were used as carbon and nitrogen source at C:N ratio 30:1. Cells were incubated at 30°C, pH 7.0, aeration rate 0.5 vvm agitation speed 200 rpm, for 120 h. The aeration rate in this experiment was constant at 0.5 vvm, in order to compare with increasing of aeration rate in topic 4.5. Because of the result indicated that aeration rate had strong effect on xanthan gum production but using high aeration rate present foam problem.

Culture medium was collected every 4 h to check growth, amylase activity, and reducing sugar content then at 120 h of fermentation xanthan gum content was detected. The results show in Fig 4.20 to 4.22, and Table 4.10.

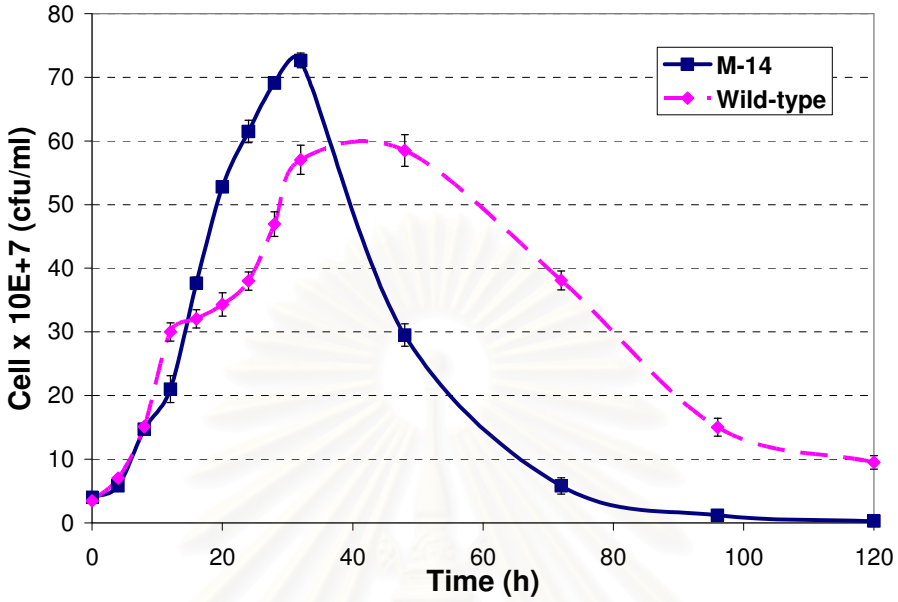


Fig 4.20 Growth curve of mutant strain and wild-type of *X. campestris* cultured in 3 liters of XOL medium contained raw cassava starch in a 5 liters bioreactor. The cells were incubated at 30°C, pH 7.0, aeration rate 0.5 vvm, agitation speed 200 rpm, for 120 h

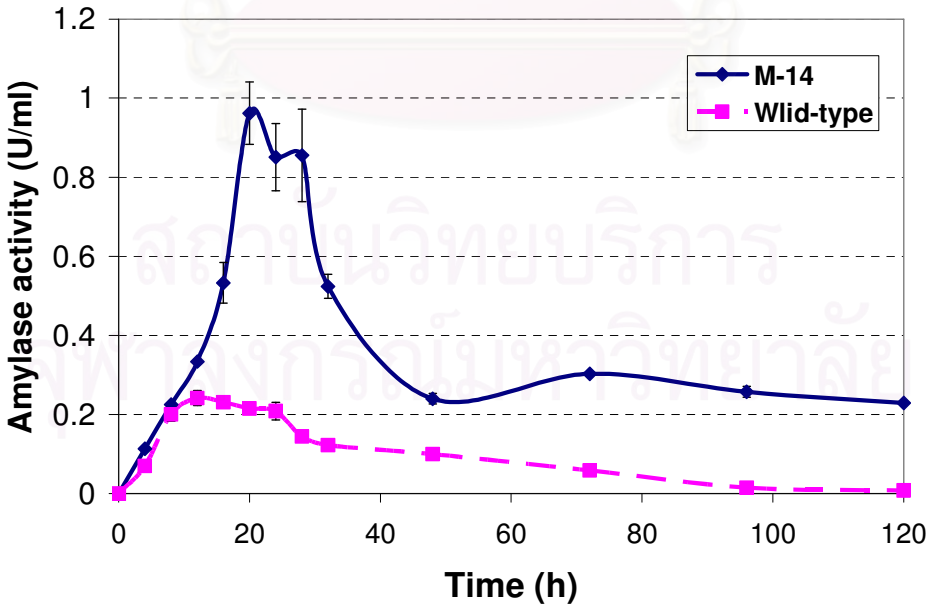


Fig 4.21 Amylase production of mutant strain and wild-type of *X. campestris* cultured in 3 liters of XOL medium contained raw cassava starch in a 5

liters bioreactor. The cells were incubated at 30°C, pH 7.0, aeration rate 0.5 vvm, agitation speed 200 rpm, for 120 h

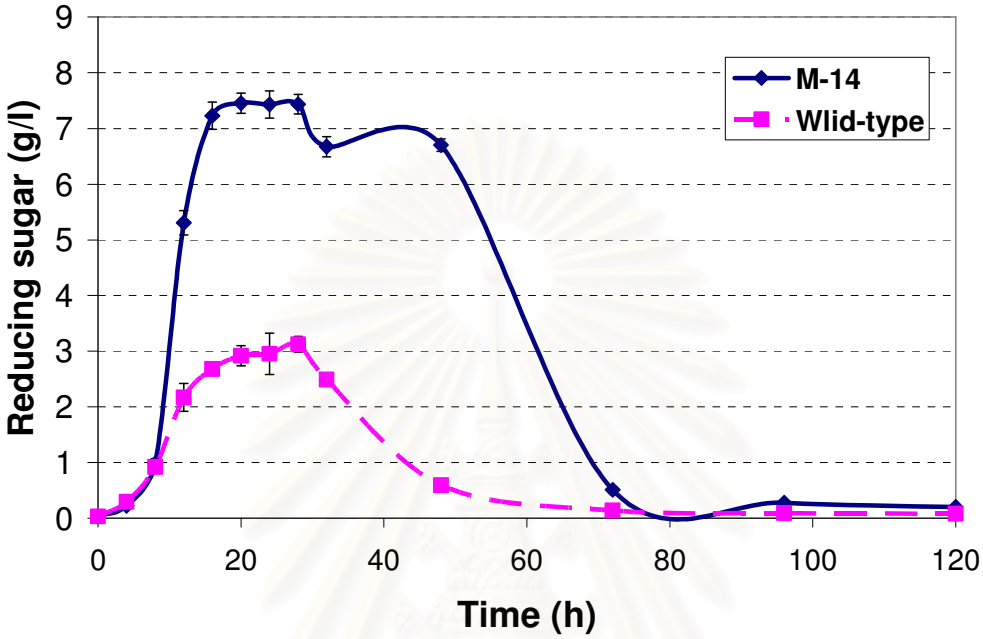


Fig 4.22 Reducing sugar content over time of fermentation of mutant strain and wild-type of *X. campestris* cultured in 3 liters of XOL medium contained raw cassava starch in a 5 liters bioreactor. The cells were incubated at 30°C, pH 7.0, aeration rate 0.5 vvm, agitation speed 200 rpm, for 120 h

Table 4.10 Amylase activity and xanthan gum production of mutant and wild-type of *X. campestris* cultured in 5 liters bioreactor

Strain	Highest amylase activity* (U/ml)	Xanthan gum* (g/l)	Viscosity* (mPa.s)
M-14	0.962 ± 0.042	5.97 ± 0.51	45.35 ± 4.42
Wild-type	0.242 ± 0.036	4.31 ± 0.13	39.63 ± 3.49

* The results were mean of 3 replications

In the XOL minimal medium, M-14 and wild-type exhibited difference of growth, amylase production, and xanthan gum production. Fig 4.20 showed that, M-14 grown very well in XOL minimal medium and higher amylase activity was detected from M-14 culture medium. The highest amylase activity (at 20 hour of cultivation) of M-14 and wild-type are showed in Table 4.9, the mutant exhibited more than 2-fold of amylase activity compare with wild-type and xanthan gum yield of M-14 was also significantly higher.

Reducing sugar over time of fermentation was observed (Fig 4.22) to explain the benefit of EMS mutagenesis of *X. campestris*. The result revealed that, very high production of amylase from M-14 (Fig 4.21) lead to high ability to hydrolyze raw cassava starch in the fermentation system. Very high reducing sugar content was detected after 8 h of cultivation, it related with high amylase production after 8 h. From this evidence, it was no doubt in higher growth and xanthan gum production of M-14 compared with wild-type.

The same result has been reported by Yang, Wu, and Tseng (2002). They created β-galactosidase overproduction strain of *X. campestris* by nitrous acid mutagenesis. The researcher found that mutant of *X. campestris* that showed very high production of β-galactosidase could use lactose base medium for xanthan gum production. The mutant produced 1.852 g/l xanthan gum, which is 20-fold higher than that produced by wild-type, 0.092 g/l, when XOL medium contain lactose as sole carbon source was used. In 1997, Rodriguez and Aguilar found that they could select mutant strain of *X. campestris* that gave overproduction of xanthan gum from the halo size on starch plate. The coordinate regulation of xanthan gum synthesis

and a series of extracellular enzymes, including amylase, have been described in *X. campestris* (Tang, Gough, and Daniels, 1990).

In this experiment, the mutant strain produced 5.97 g/l of xanthan gum. The result was significantly higher than production of the wild-type. However, increasing aeration rate from 0.5 to 1.0 vvm in the topic 4.5 could increase xanthan gum yield from 4.30 to 7.95 g/l. The result indicated that aeration rate had greater effect on xanthan gum production compared with EMS mutagenesis when culturing in the same C:N ratio. Therefore, xanthan gum production by M-14 mutant strain in culture medium containing C:N ratio at 30:1 and apply aeration rate at 1.0 vvm is interesting for a future study.

CHAPTER V

CONCLUSION

Xanthomonas campestris can grow in XOL medium containing cassava starch. In the first experiment gelatinized or raw cassava starch was used as main carbon source. Cells were grown in 5 liters bioreactor and the result showed that *X. campestris* culture in the XOL medium containing gelatinized cassava starch can produce 9.06 g/l of xanthan gum. However, the high conversion of substrate to xanthan gum indicated high impurity of xanthan gum product.

Raw cassava starch was used instead of gelatinized form to reduce impurity in xanthan gum product. The size exclusion chromatogram of HPLC showed lower impurity, or low molecular weight component, when raw cassava starch was used. It made raw form of starch more suitable using to produce xanthan gum than the gelatinized form.

Amylase produced by *X. campestris* cultured in raw cassava starch medium was partial purified by microfiltration, ultrafiltration, and dialysis. Optimum conditions of partial purified amylase produce to hydrolyze soluble starch were 40°C , and pH 7.0. The activity of partial purified amylase from *X. campestris* can reduce by adding Fe^{2+} to the reaction mixture, but Mg^{2+} , Mn^{2+}, and Ca^{2+} have no effect.

Peptone is a good supplement for growth and amylase production of bacteria. Addition of 0.125% peptone into XOL medium containing raw cassava starch as a

carbon source was significantly effected on growth and amylase production of *X. campestris*

The C:N ratio that higher than 20:1 had no effect on growth, amylase production, and xanthan gum production by *X. campestris* when the aeration rate was constant at 0.5 vvm, and raw cassava starch and ammonium sulfate were used as a carbon and a nitrogen source, respectively. Central Composite Design was used to observe the effect of C:N ratio and aeration rate respond to highest amylase production and xanthan gum yield. The increasing of highest amylase activity and xanthan gum yield were detected when C:N ratio and aeration rate was increased. The optimum condition for production of xanthan gum using raw cassava starch as carbon source was 0.88 – 1.0 vvm aeration and C:N ratio at 27:1 – 30:1.

Chemical mutation by ethyl methanesulfonate (EMS) can increase amylase production of *X. campestris*. The mutant strain was detected by observe large clear zone on starch agar plate. Growth and xanthan gum production of mutant strain were higher than found in wild-type. The mutant strain could produce 5.97 g/l of crude xanthan gum but wild-type could produce 4.31 g/l when raw cassava starch was used as a carbon source.

RECOMMENDATION

Xanthan gum production by mutant strain of *X. campestris* in production medium at the optimum condition from topic 4.5 was interesting. Moreover, properties of xanthan produced from raw cassava starch and comparison with commercial food grade xanthan should be done for future studies.

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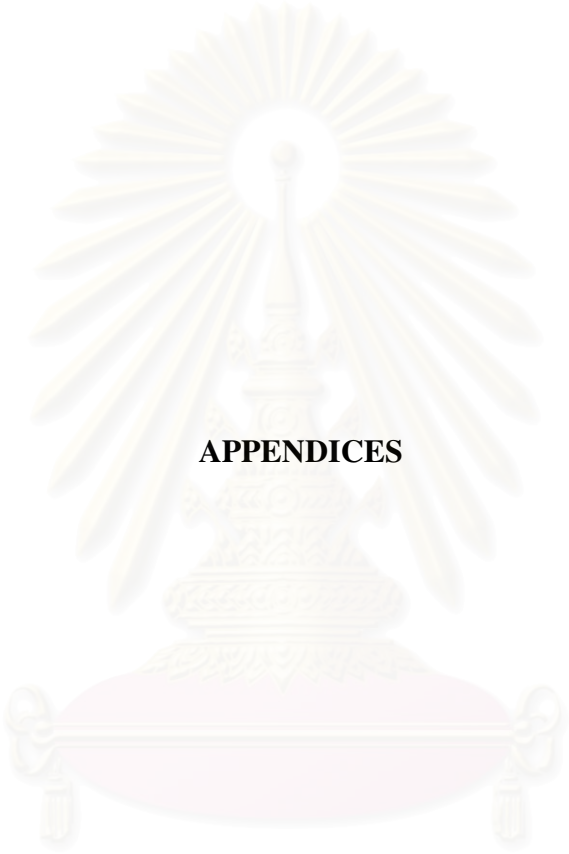


APPENDICES

APPENDIX A

CULTURE MEDIUM AND BUFFER PREPARATION

1. Yeast Malt Extract (YM) Agar

Glucose	10	g
Yeast extract	3	g
Malt extract	3	g
Peptone	5	g
Agar powder	15	g
Distilled water	1000	ml
pH	7.0 ± 0.1	

2. Yeast Malt Extract (YM) Broth

Glucose	10	g
Yeast extract	3	g
Malt extract	3	g
Peptone	5	g
Distilled water	1000	ml
pH	7.0 ± 0.1	

3. XOL minimal medium

K_2HPO_4	0.7	g
KH_2PO_4	0.2	g
$(NH_4)_2SO_4$	1.0	g
$MnCl_2$	0.001	g
$FeSO_4$	0.01	g
$MgCl_2$	0.1	g
Distilled water	1000	ml
pH	7.0 ± 0.1	

plus 0.125% peptone

4. Production medium (Roseiro *et al*., 1992) improved by Thanyaporn (1999)

Glucose	30	g
$(NH_4)_2SO_4$	1.0	g
H_3BO_3	0.0072	g
$FeCl_3$	0.0042	g
KH_2PO_4	7.2	g
$CaCO_3$	0.029	g
$MgSO_4$	0.1	g
ZnO_2	0.006	g
Citric Acid ($C_6H_8O_7$)	2.0	g
Distilled water	1000	ml
pH	7.0 ± 0.1	

5. A Buffer

K_2HPO_4	10.5	g
KH_2PO_4	4.5	g
$(NH_4)_2SO_4$	1.0	g
Sodium citrate	0.5	g
Distilled water	1000	ml
pH	7.0 ± 0.1	

6. 0.05 M Potassium phosphate buffer pH 7.0

K_2HPO_4	8.71	g/l
KH_2PO_4	6.8	g/l

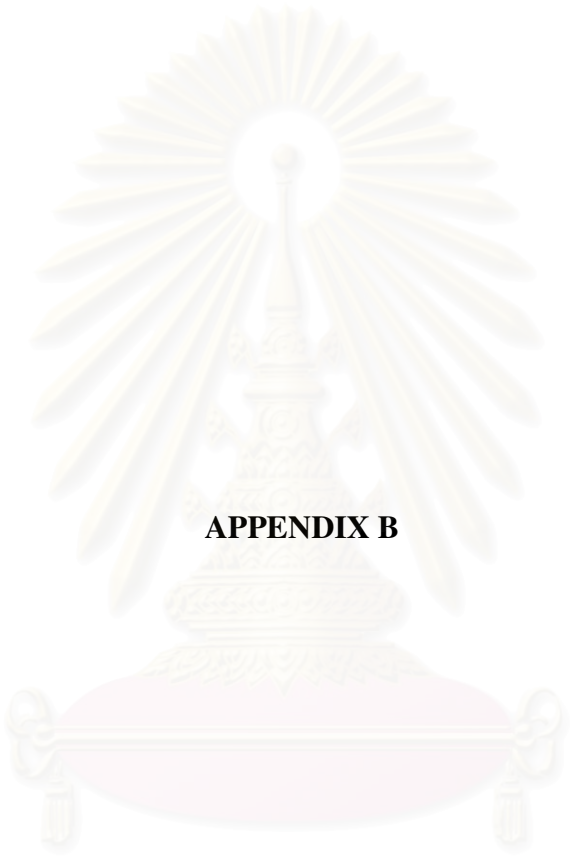
Mix K_2HPO_4 : KH_2PO_4 at 60.16 : 39.84 ml

7. McIlvaine's buffer

Solution A (0.1M Citric acid) = 21.01g/l

Solution B (0.2M Disodium hydrogen phosphate) = 35.60g/l

pH	Solution A (ml)	Solution B (ml)
3.0	4.11	15.89
4.0	7.71	12.29
5.0	10.30	9.70
6.0	12.63	7.37
7.0	16.47	3.53
8.0	19.45	0.55



APPENDIX B

ANALYTICAL METHOD

1. Dinitrosalicylic acid method (DNSA method) (Robyt and Whelan, 1965)

Reagent preparation

3,5-Dinitrosalicylic acid	12.5 g
2 N Sodium hydroxide	50 ml
Sodium potassium tartate	75 g
Distilled water	to 250 ml

The reaction mixture was prepared by dissolved 12.5 g of 3,5-dinitrosalicylic acid in 50 ml of 2 N sodium hydroxide. Then 75 g of sodium potassium tartate was added into the mixture and stirred until it completely dissolved. Distilled water was added to adjust the solution to 250 ml. The solution was place into brown bottle and kept at room temperature.

Method

The reducing sugar content in cultured medium was detected by DNSA method. After centrifuged at 8,000 rpm to remove cells, 1 ml of cultured medium was place in test tube and added 1 ml of DNSA reagent solution then boiled for 10 minute. The mixture was diluted to 10 ml with distilled water and absorbance of the resulting solution measured at 540 nm was compared to a glucose standard plot (Amritkar, Kamat, and Lali, 2004).

2. Amylase activity assay

Reagents

1% w/v of soluble starch

DNSA reagent from item 1

Method

For total amylase activity, 2 ml of crude enzyme samples were added to 0.5 ml of 1% w/v soluble starch solution in 50 mM potassium phosphate buffer pH 7.0 and the mixture was incubated for 30 minute at 35°C in a water bath. The reaction (1 ml) was stopped by addition of 1 ml of DNSA reagent solution and total reducing sugar was measured by DNSA method as described above. (Tseng and Pang, 1985). One amylase enzyme unit (EU) was taken as the amount of enzyme protein required to

release on micromole of glucose per minute under the given assay condition. Specific activity of amylase can calculate with the equation below.

$$\text{Specific amylase activity (U/mg)} = \frac{\text{Total amylase activity (U/ml)}}{\text{Total protein content (mg/ml)}}$$

3. Protease activity assay

Reagents

0.6% refined casine

0.05 M phosphate buffer (pH 7.3)

0.11 M trichloroacitic acid

0.33 M acetic acid

0.22 M sodium acetate

Method

Hydrolytic activity of protease against casein was assayed by the modified method of Uehara *et al* (1974). A 1 ml of 0.6% refined casein in 0.05 M phosphate buffer (pH 7.3) was mixed with 0.5 ml of an enzyme solution. After the mixture was incubated at 40°C for 30 min,

1.0 ml of 0.11 M trichloroacetic acid containing 0.33 M acetic acid and 0.22 M sodium acetate was added. The solution stood at room temperature for 30 min. The insoluble part of the mixture was removed by centrifugation, and the absorbance of the supernatant was measured with a spectrophotometer at 275 nm. One unit of enzyme activity was defined as that amount of enzyme which increased 0.001 in the absorbance, at 275 nm, per min at 40°C under the above conditions.

4. Crude xanthan quantification

Reagents

95% ethanol

Method

Culture broth was centrifuged at 8,000 rpm for 15 min. The amount of crude xanthan produced was determined by precipitating with three volumes of 95% ethanol (Yoo and Harcum, 1999). The dried mass was the amount of crude xanthan.

5. Determination of molecular weight of xanthan by Gel Permeation Chromatrography

Xanthan sample, 0.02 g, was dissolved in NaOH solution, pH 11.0, 10 ml. The mixture was then filtrated through the 0.4 μm nylon filter. The molecular weight of xanthan was determined by gel permeation chromatography using Ultrahydrogel Linear column and sodium hydroxide pH 11 was used as mobile phase at flow rate 0.6 ml/min. Infrared detector was used to detect the sample and the standard agent was pululan.

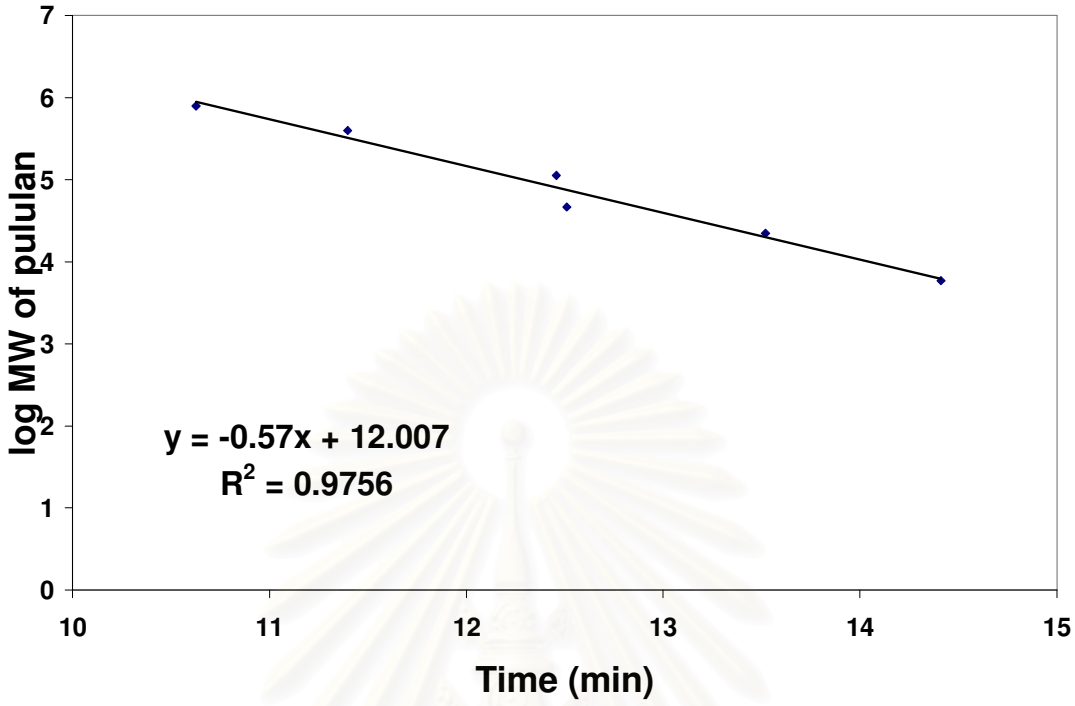


Fig B-1 standard curve of pululan for determination of molecular weight of xanthan gum

APPENDIX C

STATISTICAL ANALYSIS

1. Table of statistical analysis of effect of C:N ratio on growth of *Xanthomonas campestris* (CRD)

ANOVA

GROWTH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.079	2	2.040	.421	.661
Within Groups	116.232	24	4.843		
Total	120.312	26			

2. Table of statistical analysis of effect of C:N ratio on amylase production by *Xanthomonas campestris* (CRD)

ANOVA

AMYLASE

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.186E-03	2	1.593E-03	.877	.429
Within Groups	4.359E-02	24	1.816E-03		
Total	4.677E-02	26			

3. Table of statistical analysis of effect of C:N ratio on xanthan production by *Xanthomonas campestris* (CRD)

ANOVA

XANTHAN

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	181.859	2	90.929	3.917	.034
Within Groups	557.196	24	23.217		
Total	739.055	26			

XANTHAN

Duncan[a]

CN	N	Subset for alpha = .05	
		1	2
10.00	9	1.58767	
20.00	9	5.70933	5.70933
30.00	9		7.84000
Sig.		.082	.358

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 9.000.

4. Table of statistical analysis of effect of aeration rate on growth of *Xanthomonas campestris* (CRD)

ANOVA

GROWTH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	110.145	2	55.073	130.012	.000
Within Groups	10.166	24	.424		
Total	120.312	26			

GROWTH

Duncan[a]

AIR	N	Subset for alpha = .05		
		1	2	3
.00	9	.501333		
.50	9		3.430000	
1.00	9			5.418889
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 9.000.

5. Table of statistical analysis of effect of aeration rate on amylase production by *Xanthomonas campestris* (CRD)

ANOVA

AMYLASE

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.299E-02	2	1.649E-02	28.718	.000
Within Groups	1.378E-02	24	5.743E-04		
Total	4.677E-02	26			

AMYLASE

Duncan[a]

		Subset for alpha = .05		
AIR	N	1	2	3
.00	9	.205211		
.50	9		.254144	
1.00	9			.290522
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 9.000.

6. Table of statistical analysis of effect of aeration rate on xanthan production by *Xanthomonas campestris* (CRD)

ANOVA

XANTHAN

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	440.369	2	220.185	17.692	.000
Within Groups	298.686	24	12.445		
Total	739.055	26			

XANTHAN

Duncan[a]

AIR	N	Subset for alpha = .05	
		1	2
.00	9	1.36833	
.50	9	3.09978	
1.00	9		10.66889
Sig.		.308	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 9.000.

7. Table of statistical analysis of effect of C:N ratio and aeration rate on growth of *Xanthomonas campestris* (factorial 3 x 3)

Dependent Variable: GROWTH

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	119.211[a]	8	14.901	243.724	.000
Intercept	262.280	1	262.280	4289.790	.000
CN	4.079	2	2.040	33.361	.000
AIR	110.145	2	55.073	900.756	.000
CN * AIR	4.986	4	1.247	20.389	.000
Error	1.101	18	6.114E-02		
Total	382.592	27			
Corrected Total	120.312	26			

a. R Squared = .991 (Adjusted R Squared = .987)

8. Table of statistical analysis of effect of C:N ratio and aeration rate on amylase production by *Xanthomonas campestris* (factorial 3 x 3)

Dependent Variable: AMYLASE

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4.306E-02[a]	8	5.382E-03	26.082	.000
Intercept	1.687	1	1.687	8175.159	.000
CN	3.186E-03	2	1.593E-03	7.720	.004
AIR	3.299E-02	2	1.649E-02	79.930	.000
CN * AIR	6.884E-03	4	1.721E-03	8.340	.001
Error	3.714E-03	18	2.064E-04		
Total	1.734	27			
Corrected Total	4.677E-02	26			

a. R Squared = .921 (Adjusted R Squared = .885)

9. Table of statistical analysis of effect of C:N ratio and aeration rate on xanthan production by *Xanthomonas campestris* (factorial 3 x 3)

Dependent Variable: XANTHAN

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	735.415[a]	8	91.927	454.584	.000
Intercept	687.386	1	687.386	3399.165	.000
CN	181.859	2	90.929	449.651	.000
AIR	440.369	2	220.185	1088.825	.000
CN * AIR	113.187	4	28.297	139.929	.000
Error	3.640	18	.202		
Total	1426.441	27			
Corrected Total	739.055	26			

a. R Squared = .995 (Adjusted R Squared = .993)

10. Table of statistical analysis of effect of ethylmethan sulfonate (EMS) on growth of *Xanthomonas campestris* (CRD)

ANOVA

GROWTH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7.504	1	7.504	138.696	.000
Within Groups	.216	4	5.410E-02		
Total	7.720	5			

11. Table of statistical analysis of effect of ethylmethan sulfonate (EMS) on amylase production of *Xanthomonas campestris* (CRD)

ANOVA

AMYLASE

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.856	1	.856	1432.689	.000
Within Groups	2.389E-03	4	5.973E-04		
Total	.858	5			

12. Table of statistical analysis of effect of ethylmethan sulfonate (EMS) on xanthan production of *Xanthomonas campestris* (CRD)

ANOVA

XANTHAN

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	37.700	1	37.700	4181.176	.000
Within Groups	3.607E-02	4	9.017E-03		
Total	37.736	5			

13. Tables of statistical analysis of central composite design of highest amylase activity of *Xanthomonas campestris* cultured in 5 liter bioreactor

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.946[a]	.895	.878	.013034

a. Predictors: (Constant), CN_AIR, AIR2, AIR, CN, CN2

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	.044	5	.009	51.379	.000[a]
	Residual	.005	30	.000		
	Total	.049	35			

a. Predictors: (Constant), CN_AIR, AIR2, AIR, CN, CN2

b. Dependent Variable: AMYLASE

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	.250	.003		72.832	.000
	CN	.011	.003	.210	3.562	.001
	AIR	.046	.003	.877	14.846	.000
	CN2	-.004	.005	-.059	-.940	.355
	AIR2	-.001	.005	-.009	-.145	.886
	CN_AIR	.018	.004	.281	4.762	.000

a. Dependent Variable: AMYLASE

14. Tables of statistical analysis of central composite design of highest amylase activity of *Xanthomonas campestris* cultured in 5 liter bioreactor

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.994[a]	.988	.986	.25943

a. Predictors: (Constant), CN_AIR, AIR2, AIR, CN, CN2

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	163.566	5	32.713	486.049	.000[a]
	Residual	2.019	30	.067		
	Total	165.585	35			

a. Predictors: (Constant), CN_AIR, AIR2, AIR, CN, CN2

b. Dependent Variable: XANTHAN

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	4.018	.068		58.765	.000
	CN	1.239	.061	.408	20.260	.000
	AIR	2.527	.061	.833	41.320	.000
	CN2	-.759	.092	-.177	-8.277	.000
	AIR2	-.172	.092	-.040	-1.881	.070
	CN_AIR	1.110	.075	.299	14.822	.000

a. Dependent Variable: XANTHAN

VITAE

Miss Paramaporn Kerdsup was born on April 15, 1978 in Bangkok, Thailand. She obtained a B.Sc. degree in Food Technology from Chulalongkorn University in 1998 and M.Sc. degree in Food Technology from Chulalongkorn University in 2002. She started the first job as a lecturer at Bansomdejchaopraya Rajabhat University, Thailand during 2002-2004. In 2004, she started her Ph.D. studies at the Biotechnology Program, Chulalongkorn University. After being finished her Ph.D. degree, she is going to join the Institute for Innovation and Development of Learning Process at Mahidol University.