

การคัดแยกเชื้อแบคทีเรียที่ผลิตเอนไซม์อะไมเลสจากน้ำเสียโรงงานแป้ง

## ISOLATION OF AMYLASE PRODUCING BACTERIA FROM STARCH INDUSTRIAL WASTEWATER

อำพรณ ชัยกุลเสรีวัฒน์<sup>1</sup> และชนิดา โทบูน

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**บทคัดย่อ:** ศึกษาการคัดแยกเชื้อแบคทีเรียที่ผลิตเอนไซม์อะไมเลสจากน้ำเสียโรงงานแป้ง ในทดลองนี้สามารถคัดแยกเชื้อได้ 5 สายพันธุ์ที่สามารถสร้างวงใสรอบๆ โคลนินของเชื้อ เมื่อเจริญบนอาหาร starch agar พบเชื้อที่มีความสามารถในการผลิตเอนไซม์อะไมเลสได้สูงมี 2 สายพันธุ์ คือสายพันธุ์ A และ C โดยผลิตเอนไซม์ได้  $0.36 \pm 0.04$  และ  $1.30 \pm 0.03$  ยูนิต ที่อุณหภูมิ 37 องศาเซลเซียส ในเวลา 24 ชั่วโมง โดยที่ทั้ง 2 สายพันธุ์นี้มีรูปร่างเป็นแท่ง ดิคลิแกรมบวก และเป็นแบคทีเรียที่สร้างเอนโดสปอร์ได้ จากนั้นศึกษาชนิดของแหล่งคาร์บอน ค่าความเป็นกรด-เบสเริ่มต้น และอุณหภูมิที่เหมาะสมในการผลิตเอนไซม์อะไมเลส พบว่า เชื้อสายพันธุ์ A และ C สามารถผลิตเอนไซม์อะไมเลสได้สูงสุดโดยมีกิจกรรมของเอนไซม์เท่ากับ  $1.54 \pm 0.06$  และ  $1.80 \pm 0.09$  ยูนิต เมื่อเลี้ยงเชื้อในอาหารที่มี องค์ประกอบของแป้งมันสำปะหลัง ค่าความเป็นกรด-เบส เท่ากับ 7 และบ่มที่อุณหภูมิ 37 องศาเซลเซียส เป็นเวลา 24 ชั่วโมง

**Abstract:** This study was investigated the isolation of amylase producing bacteria from a starch industrial wastewater sample. In the present study five strains of amylase producing microorganism were isolated. The organisms were capable to produce halo-forming zone around their colonies on starch agar medium. There were two strains (strain A and C) that had amylase activities. The strain A and C showed good amylase activities at 37 °C for 24 hrs as  $0.36 \pm 0.04$  and  $1.30 \pm 0.03$  U, respectively. The both strains were Gram positive bacilli and endospore forming bacteria. After that, the effect of various carbon sources, initial medium pH and incubation temperature on amylase production were investigated. The maximum amylase activity of the strain A and C ( $1.54 \pm 0.06$  and  $1.80 \pm 0.09$  U) in medium containing cassava flour were obtained at an initial medium pH 7, while the incubation temperature investigated was 37°C after 24 hrs of incubation.

**Introduction:** Amylases are enzymes which hydrolyse starch molecules to give diverse products including dextrans and progressively smaller polymers composed of glucose units [1]. These enzymes have a great significance with extensive biotechnological applications in bread and baking, food, textile, and paper industries. Among various extracellular enzymes, amylase ranks first in terms of commercial exploitation [2]. Spectrum of applications of amylase has widened in many sectors such as clinical, medicinal and analytical chemistry. Besides their use in starch saccharification, they also find applications in baking, brewing, detergent, textile, paper and distilling industry [3]. Amylases are universally distributed throughout the animal, plant and microbial kingdoms. However, enzymes from fungal and bacterial sources have dominated applications in industrial sectors. Several *Bacillus* sp. and thermostable actinomycetes like thermomonospora and thermoactinomyces are versatile producers of the enzyme [4]. The genus *Bacillus* produces a large range of extracellular enzymes, of which amylases and proteases are of significant industrial importance [5]. The industrially important *Bacillus* strains are *B. licheniformis*, *B. amyloliquefaciens* [6], *B. circulans* [7] and *B. subtilis* [8]. Therefore, the objectives of the study were to isolate amylase producing bacteria from the starch industrial wastewater, and to investigate the effects of carbon sources, temperature and pH on the production of amylase.

## **Materials and methods :**

### **Isolation & screening of bacteria**

The amylase producing bacteria were isolated from a starch industrial wastewater sample (Fashion Food Co., Ltd.) by appropriate dilutions with saline, plated on starch agar plates containing 0.5% peptone, 0.2% yeast extract, 3% soluble starch, 0.13%  $\text{KH}_2\text{PO}_4$ , 0.05%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01%  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  and 1.5% agar. These plates were incubated at 37°C for 24-48 hrs. Several amylase-producing bacterial colonies were selected after flooding the plates with iodine solution (2%  $\text{I}_2$  and 0.2% potassium iodide). The strain that yielded a high level of amylase was selected for further experiments. Typical cultural and morphological characteristics were observed. The isolated strains examined by Gram staining and endospore staining [9]. The amylase producing strains were maintained on nutrient agar slant and were stored at 4°C for further studies.

### **Production of Amylase**

One hundred ml nutrient broth was inoculated with a loop-full of growing culture of isolated strain and was incubated at 37°C for 24 hrs. The 0.5 ml culture ( $\text{Abs}_{660}=0.5$ ) was transferred into the 100 ml starch broth medium (SB medium) and was incubated at 37°C for 24 hrs with shaking on a shaker (180 rpm). After incubation, the supernatant of the culture after centrifugation (4,500 rpm, 15 min) at 4°C was used to determine extracellular amylase activity [10].

## **Enzyme Assay**

The reaction mixture containing 0.5 ml of supernatant and 0.5 ml (1.0%) solution of soluble starch in 0.1 M sodium phosphate buffer (pH 7.0). After incubation at 70°C for 3 minutes, the reaction was stopped by the addition of 1 ml of 3-5-dinitrosalicylic acid reagent and 10 ml distilled water and absorbance was measured in a spectrophotometer [10]. One unit (U) is defined as the amount of enzyme which releases 1 µg of reducing end groups per minute in 0.1 M sodium phosphate buffer (pH 7.0) with 1.0 % (w/v) soluble starch as substrate during 3 minutes incubation at 70°C.

## **Effects of carbon sources on amylase production**

To determine the effect of carbon sources on amylase production. The enzyme assay was performed after pre-incubation at 37°C for 24 hrs, of the enzyme with various carbon sources each at a concentration of 3%. The enzyme assay was carried out in the presence of cassava flour, wheat flour, corn flour, rice flour and glutinous rice flour. The enzyme activity (U) were determined as described earlier.

## **Effects of initial pH on amylase production**

The effect of initial pH on amylase production was performed at pH 3-11. The pH of the medium was adjusted using 0.2 M sodium citrate (pH 3-6), 0.2 M sodium phosphate (pH 6-8) and 0.2 M glycine-NaOH (pH 8-11) [11]. The medium flasks were incubated at 37°C for 24 hrs. The enzyme activity (U) were determined as described earlier.

## **Effects of temperature on amylase production**

The effect of temperature was evaluated by incubating the reaction mixtures (100 ml of sterile production medium was prepared and inoculated with the 0.5 ml isolated strains ( $Abs_{660}=0.5$ )) at different temperatures (30, 37 and 45 °C) for 24 hrs. The enzyme activity (U) were determined as described earlier.

## **Results, Discussion and Conclusion:**

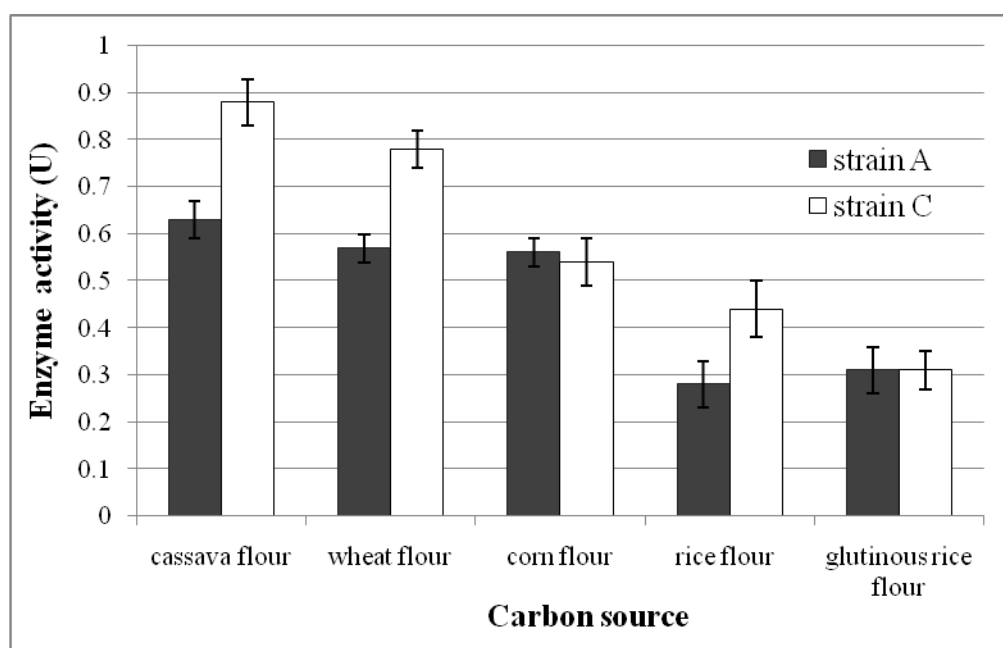
### **Isolation & screening of bacteria**

According to the results, the five isolated strains were isolated from a starch industrial wastewater sample (Fashion Food Co., Ltd.). The five strains (strain A, B, C, D and E) capable of producing alpha amylase were screened by allowing them to grow for 24 hrs on starch agar plates containing 3% starch at 37°C. The plates were stained with Gram's iodine solution (2% I<sub>2</sub> and 0.2% potassium iodide), and halo-forming zone was considered as the most promising strain. The strain A and C showed the largest halo-forming zone and were chosen for further investigation. The both strains were Gram positive bacilli, endospore forming bacteria. And then, the amylase production of

the both strains into the starch broth medium (SB medium) were studied at 37 °C for 24 hrs of incubation. The results found that the strain A and C showed amylase activity as  $0.36\pm 0.04$  and  $1.30\pm 0.03$  U, respectively. Oyeleke and Oduwole, (2009) found a comparable trend in amylase production by *Bacillus* species were isolated and identified from the cassava dumpsites [12]. The result also agrees with Oguntimehin (1998) for amylase production in *Bacillus licheniformis*, which was isolated from cassava during fermentation [13].

### Effects of carbon sources on amylase production

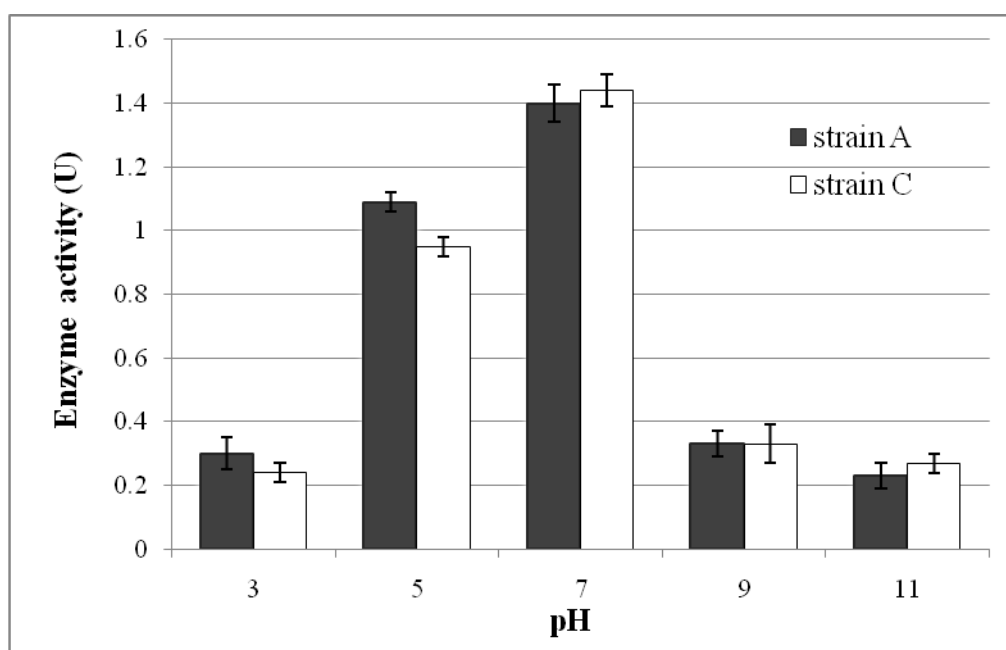
Natural sources could serve as economical and readily available raw material for production of valuable enzymes. In this study, several natural sources were used as substrates (cassava flour, wheat flour, corn flour, rice flour and glutinous rice flour). The results from Figure 1 revealed that the maximum amylase production of strain A and C on cassava flour showed amylase activity as  $0.63\pm 0.04$  and  $0.88\pm 0.05$  U, respectively. Not only the cassava but also the crude medium prepared using wheat flour also shown high amylase yield.



**Figure 1** Effect of carbon sources on amylase production by the isolated strains for 24 hrs of incubation.

### Effects of initial pH on amylase production

Enzyme synthesis of the both strains were observed between pH 3 to 11 (Fig.2). The results suggested that there were a stimulation of enzyme synthesis at pH 7 and the highest enzyme production as  $1.40\pm 0.06$  and  $1.44\pm 0.05$  U for strain A and strain C, respectively. Increasing the initial pH of the medium up to pH 9 and 11 resulted in a decrease of the amylase production. pH is one of the important factors of the growth and enzyme secretion of microorganisms as they are sensitive to the concentration of hydrogen ions present in the medium. Oguntimehin (1998) reported the maximum activities at a pH range of 4.8 and 9.2 for an amylase obtained for *B. licheniformis* isolated from a cassava processing waste [13]. Sudharhsan et al. (2007) reported that the maximum amylase production was achieved at neutral pH 7 by *Bacillus* sp., although pH 4.0–9.0 supported amylase production [14].

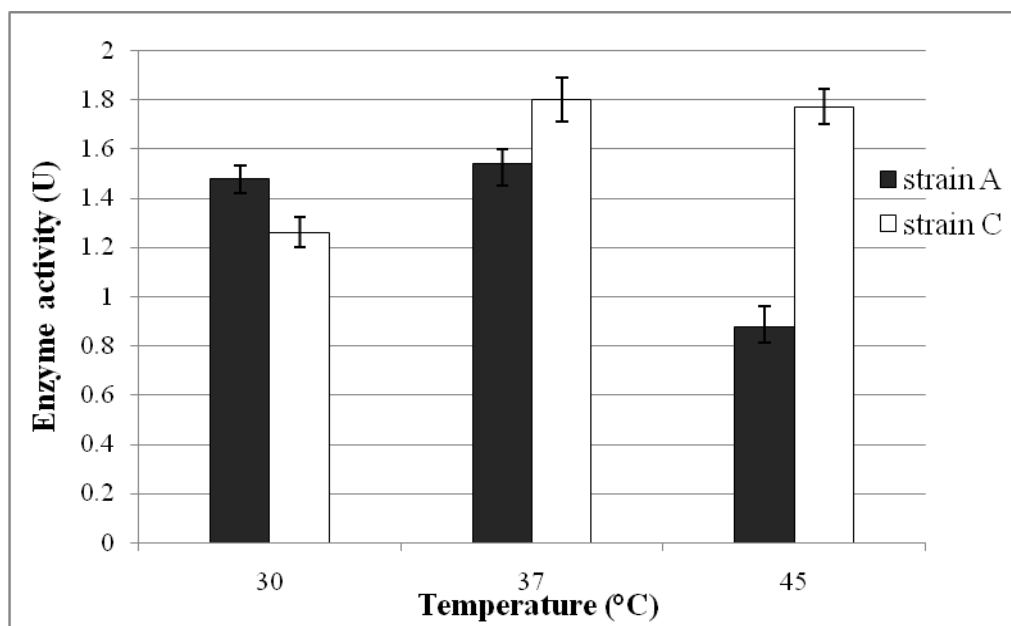


**Figure 2** Effect of pH on amylase production by the isolated strains for 24 hrs of incubation.

### Effects of temperature on amylase production

Enzyme synthesis of the both strains were determined at different temperatures ranging from 30°C to 45°C (Fig. 3). The results suggest that the optimum enzyme production were observed at 37°C and the highest enzyme production as  $1.54\pm 0.06$  and  $1.80\pm 0.09$  U for strain A and strain C, respectively. Sudharhsan et al. (2007) reported that the optimum temperatures for amylase production and growth of *Bacillus* sp. were observed at 37°C [14]. Nusrat and Rahman (2007) reported that *Bacillus* isolates was

found to grow and produce  $\alpha$ -amylase at temperatures from 30 to 50°C. Maximum enzyme production was observed at 37°C [15].



**Figure 3** Effect of temperature on amylase production by the isolated strains for 24 hrs of incubation.

In conclusion, amylase producing bacteria was isolated from the starch industrial wastewater. The various factors affecting amylase production was assayed which include carbon source, pH, and temperature. The results showed that cassava flour was found to be a best carbon source for amylase production. pH 7.0 and 37°C were found to be optimum values for maximum enzyme production. In the future, these bacteria may use to treat the agricultural waste material.

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**Keyword :** Amylase producing microorganism, Starch industrial wastewater, Amylase activity

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