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Enhanced Production of Extracellular Asparaginase in *Erwinia Carotovora* subsp. *Carotovora*

Erwinia Carotovora subsp. *Carotovora* تحسين انتاج انزيم الاسباراجينيز خارجيا من بكتيريا

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Abstract

Bacterial asparaginases are enzymes that catalyze the hydrolysis of asparagine to aspartic acid. For the past 30 years, these enzymes have been used as therapeutic agents in the treatment of acute childhood lymphoblastic leukemia.

Morphological and biochemical characteristics confirmed that the bacterium used in this study is identical to *Erwinia carotovora* subsp. *carotovora*. For cell growth of *E. carotovora* subsp. *carotovora*, the optimal pH and temperature were pH 9 and 40°C, respectively. *Erwinia carotovora* subsp. *carotovora* showed its maximal extracellular asparaginase activity at pH 7 and temperature 40°C.

Supplementation of asparagine and aspartic acid to culture medium was carried out to determine the effect of cell growth and asparaginase activity. After 12-hr cultivation, both cell growth and the enzyme activity with asparagine were enhanced, compared to no addition and aspartic acid. This results suggest that addition of asparagine might stimulate the production of extracellular aparaginase in *Erwinia carotovora* subsp. *carotovora*.

Keywords: *Asparaginase, Erwinia Caratovora, Bacteria, Enzyme, Neslar reagent.*

الخلاصة

الاسباراجينز هي انزيمات بكتيرية تعمل على تحفيز وتحليل الاسباراجين الى حامض الاسبارتك. خلال 30 سنة الماضية استخدمت هذه الانزيمات كعوامل علاجية في معالجة سرطان الدم اللمفاوي الحاد لدى الاطفال.

تؤكد الخصائص الشكلية والبايولوجية بأن البكتيريا المستخدمة في هذه الدراسة هي *Erwinia Carotovora* subsp. مطابقة *Carotovora* .

بالنسبة لنمو الخلية لل PH الامثل ودرجة الحرارة المثلى كانت 9 و40 بالتتابع وكانت الفعالية العظمى لإنزيم الاسباراجينز هي 7 و 40 على التوالي. تم تزويد الوسط الزرعي بالاسباراجينز وحامض الاسبارتك لتحديد تأثيرها على كل من نمو الخلية والفعالية الانزيمية . بعد 12 ساعة حضانة مع اضافة الاسباراجين لوحظ التحسين في نمو الخلية وفعالية الانزيم اذا ما قورنت مع العينات قبل الاضافة وعينات حامض الاسبارتك . من هذه النتائج نقترح بأن اضافة الاسباراجين ربما تحفز انتاج انزيم الاسباراجين خارجيا.

1. Introduction

Asparaginase (asparagine amino hydrolase, EC 3.5.1.1) has long being considered in the management of childhood acute lymphoblastic leukemia (ALL). Its antileukemic effect is attributed to the inability of neoplastic blast cells to synthesize asparagine from aspartic acid as they lack asparagine synthetase.

However, lymphatic tumor cells need large amounts of asparagine in order to achieve rapid malignant growth. Therefore, the commonest therapeutic practice to treat this condition is to intravenously administer asparaginase to deplete the level of blood asparagine and exhaust its supply to selectively affect the neoplastic cells [1]. Asparaginase is known to be produced from variety of organisms like *E. coli* [2], *Erwinia* species [3], *Proteus vulgaris* [4], *Serratia marcescens*

[5], *Enterobacter aerogenes* [6], *Staplylococcus* species [7], *Pseudomonas aeruginosa* [8], and *Thermus thermophilus* [9]. The reports on some filamentous fungi like *Aspergillus niger* [10].

Erwinia carotovora is a Gram-negative [11], rod-shaped bacterium that lives alone or aggregates into pairs and chains. Non-spore forming and peritrichously flagellated, it is a facultative that is catalase negative and oxidase positive. *Erwinia carotovora* produces a number of extracellular plant cell wall degrading enzymes such as pectic enzymes that degrade pectin, cellulase, hemicellulase, arabanase, cyanose and protease.

2. Materials and Methods

Chemicals and Media

Asparagine, aspartic acid, Nessler's reagent, Bradford reagent, ammonium sulfate, trichloroacetic acid (TCA) and other media components (Table 1, yeast ex., peptone, beef ex., glucose etc) was purchased from Sigma Aldrich Co. (USA).

Bacteria used and General culture conditions

Erwinia carotovora subsp. *carotovora* KCCM11319 used in this study was purchased from Korean Federation of Culture Collections (KFCC). The stock culture was maintained at 4°C and transferred weekly on nutrient agar. The plates of nutrient agar were incubated at 30°C for 48 hr and bacterial colonies were isolated from the cultivated nutrient agar. Each of the colonies was sub-cultured on nutrient agar slants.

Inoculum was prepared in 250-ml flasks containing 50 ml of Luria-Bertani medium(LB), (pH 7.0) [12]. The medium was autoclaved at 121 ºC (15 psi) for 20 min and 2-loops of *Erwinia carotovora* subsp. *carotovora* was inoculated to LB broth. The cells was cultivated on a reciprocal shaker (180 rpm) for 24 hr and were used as an inoculum.

General characteristics of *Erwinia carotovora* subsp. *carotovora*

Morphological and biochemical characteristics of *Erwinia carotovora* subsp. *carotovora* were carried out to confirm and identify. Gram's reaction, catalase test, oxidase test, nitrate reductase test were performed using a standard protocol [12], [13].

Determination of cell growth

The cells were grown at various pH and temperature in YG broth. After cultivation in 250-ml flask, 3 ml of cells was collected and their optical density at 660 nm (OD_{660}) was turbidometrically measured.

Determination of asparaginase activity

Asparaginase activity was determined by the modified method of [14] using Nessler's reaction.

The enzyme reaction and determination were as follows;

1. Preparation of Nessler's reagent
2. Preparation of the crude enzyme solution of extracellular asparaginase
3. Reaction of asparaginase in 3-ml reaction tube
4. Determination of asparaginase activity

Determination of ammonium concentration

Prepare a series of ammonium sulfate diluted with 0.15 M NaCl to give a final concentrations of 0 (blank = NaCl only), 5, 10, 12, 14, 16, 18, 20 and 25 mg/ml. After Nessler reaction, measure the absorbance at 450 nm.

Determination of protein concentration

Protein concentration was determined by the method of Bradford. [15] using a bovine serum albumin (BSA) as a standard. Prepare a series of standard protein solution diluted with 0.15 M NaCl to give a final concentrations of 0 (blank = NaCl only), 200, 400, 600, 800 and 1000 µg/ml. Also prepare serial dilutions of the unknown sample to be measured. Add 50 µL of each of the above to a separate test tube and add 3.0 mL of Coomassie brilliant blue solution to each tube and mix by

vortex. Adjust the spectrophotometer to a wavelength of 595 nm, and blank using the tube which contains DW. Wait 5 min and read each of the standards and each of the samples at wavelength of 595 nm. Plot the absorbance of the standards vs. their concentration. Calculate the concentrations of the unknown samples [16] Bradford. from the standard curve of protein. The enzyme samples were processed in a similar manner as described above.

Effect of pH and temperature on cell growth and asparaginase activity

Effect of pH and temperature on cell growth and enzyme activity of *E. carotovora* subsp. *carotovora* was determined. Two loops of cells from a slant was inoculated to YG culture broth (100 ml) in a 250-ml flask. The cells were grown at various pH (ranged in pH 3 to 12) and temperature (20 to 70°C) and every 2-hr sample (3 ml) was collected for 12 hr and OD_{660} was measured.

Addition of aspargine and aspartic acid for determination of cell growth and asparaginase activity

In order to determine the effect of asparagine and aspartic acid on cell growth and enzyme activity of *E. carotovora* subsp. *carotovora*, 3% of asparagine and aspartic acid was added to YG broth (yeast ex 0.5 g) and glucose (2 g) per l of DW, pH 9.0). Cells were grown at 40 °C and in pH 9.0 for 24 hr. Determination of cell growth and asparaginase activity was used by the method mentioned previously.

3. Results and Discussion

Morphological, biochemical and physiological characterization of *E. carotovora* subsp. *carotovora*

The bacterium used in this study was characterized on the basis of its growth characteristics, morphological and biochemical properties such as Gram's reaction, catalase test, oxidase test, nitrate reductase test using a standard protocol as shown in (Table 2).

E. carotovora subsp. *carotovora* (Fig. 2) was gram negative and rod-shaped bacterium [12],[17] and showed positive oxidation-fermentation test (Fig. 3), nitrate reductase test (Fig. 4), oxidase test (Fig. 5), negative catalase activity. When *E. carotovora* subsp. *carotovora* used in this study was infected to potato, it caused the soft black disease in the potato (Fig. 6), same as the results of many reports [12],[18],[19],[20].

When E. carotovora subsp. carotovora was cultivated at 40 C for 48 hr. in the YG broth (pH 9), cell growth reached maximum in 24 hr (Fig. 7).

Effect of initial pH and temperature on cell growth

The pH effect on cell growth was carried out ranged in pH 3 to 12 and temperature in 5 to 70. The optimal pH and temperature for cell growth were 9 (Fig. 8) and 40℃ (Fig. 9), respectively. Similar results have been reported for *Bacillus* sp. [21], *Erwinia carotovora* [22] and marine actinomycetes [23].

Effect of initial pH and temperature on extracellular asparaginase activity

The pH effect on the enzyme activity was carried out ranged in pH 3 to 12 and temperature in 20 to 70°C. The optimal pH and temperature for cell growth were 7 (Fig. 10) and 40°C (Fig. 11), respectively. This result is similar to the reports of *Corynebacterium glutamicum* [24] and *Streptomyces gulbargensis* [25].

Addition effect of asparagine and aspartic acid on cell growth and extracellular asparginase actity

To determine the effect of asparagine addition, cells were grown at 40°C for 24 hr in YG broth (pH 9) supplemented with various concentration of each amino acid. Althought over 3% additions of asparagine and aspartic acid were much better for cell growth (Fig. 12), 3-5% additions of asparagine were best in 12 hr cultivation (Fig. 13).

Time course of cell growth and extracellular asparaginase actity under optimized conditions

To compare cell growth and enzyme acitivity, cells of *E. carotovora* subsp. *carotovora* were grown at 40°C for 12 hr in YG broth (pH 9) supplemented with 3% of each asparagine and aspartic acid. Much better cell growth and enzyme activity were shown in aspargine supplemented broth compare to aspartic acid and no addition (Fig. 14, 15, 16). The activities of with asparagine, aspartic acid and no addition were 2649.5, 2069.8 and 2078.2 unit (Table 3). These are similar to the results reported by *Erwinia carotovora* [22], *Enterobacter cloaca* [26] and *Streptomyces gulbargensis* [25].

Also, extracellular protein production was enhanced, as asparagine was added to the culture broth (Fig. 17). The protein concentrations produced with aspargine, aspartic acid and no addition were 389.5, 280.3 and 364.3 mg/l, respectively (Table 3).

Table 1. Composition of complex media used in this study

Component	Concentration (%, w/v)	
	Luria Bertani	Yeast extract Glucose
Peptone	1	
NaCl	1	
Yeast extract	0.5	0.5
Glucose		2

Table 2. Biochemical and physiological characterization of *E. carotovora* subsp. *carotovora*

Characteristics	Results
Growth temperature	37-40°C
Optimal pH	9
Growth in the nutrient agar	+
Growth in the 15% NaCl (LB agar)	+
Methylene blue staining	+
Gram reaction	Negative
Oxidase test	+
Nitrate reductase test	+
Oxidation-Fermentation test	+
Catalase	-
Case the soft disease in the potato	+

Table 3. Extracellular asparaginase activity, protein concentration and specific activity in *E. carotovora* subsp. *Carotovora*

Samples	Asparginase activity unit (µmole/l)	Protein concentration (mg/l)	Specific activity (unit/mg・protei n)
No addition	2,078.2	364.3	5.70
Aspartic acid	2,069.8	280.3	7.39
Asparagine	2,649.53	389.5	6.80

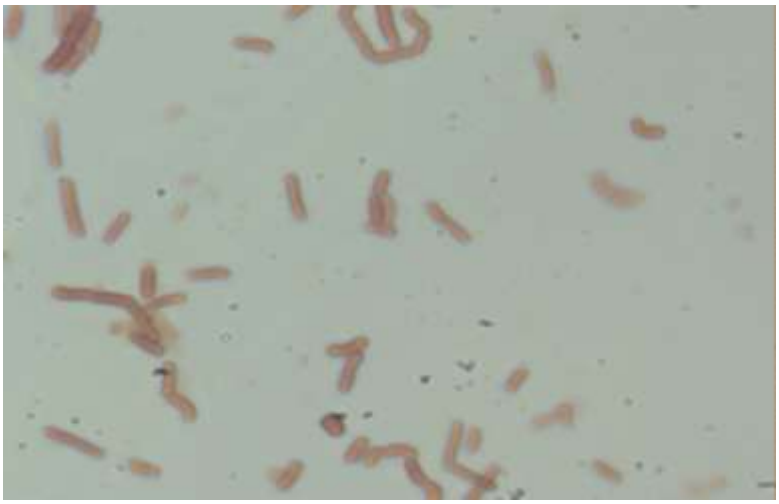

Fig. 2. Gram staining of Erwinia carotovora subsp. carotovora. Cells were shown in pink, means Erwinia carotovora subsp. carotovora is Gram-negative.

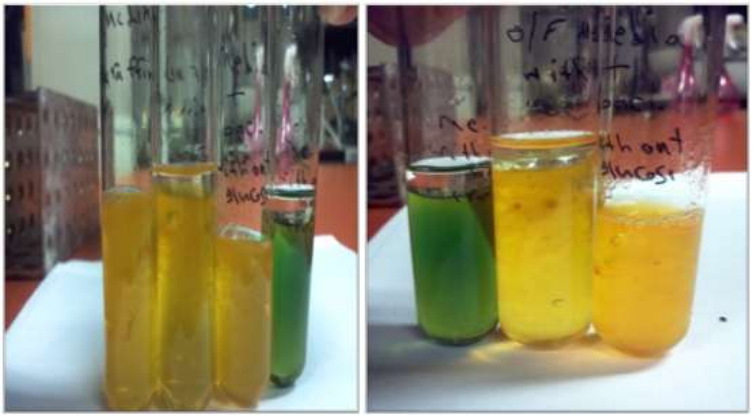

Fig. 3. Oxidation-fermentation test of Erwinia carotovora subsp. carotovora, which is O/F positive.

Fig. 4. Nitrate reduction test of *Erwinia carotovora* subsp. *carotovora.,* which means nitrate reductase positive.

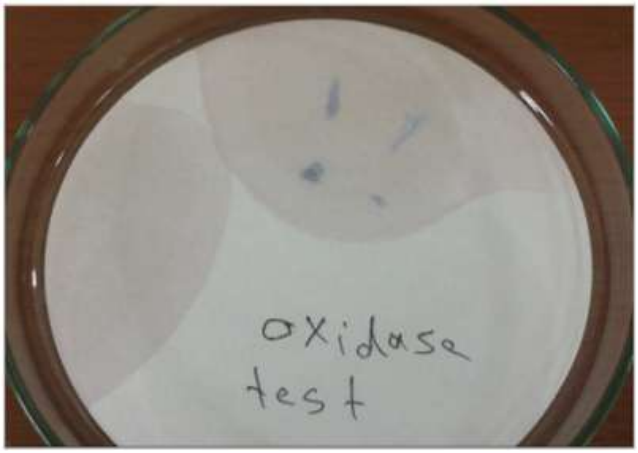

Fig. 5. Cytochrome c oxidase test of Erwinia carotovora subsp. carotovora., which means oxidase positive.

Fig. 6. The soft disease in potato caused by *Erwinia carotovora* subsp. *carotovora*, which causes soft black disease of potato.

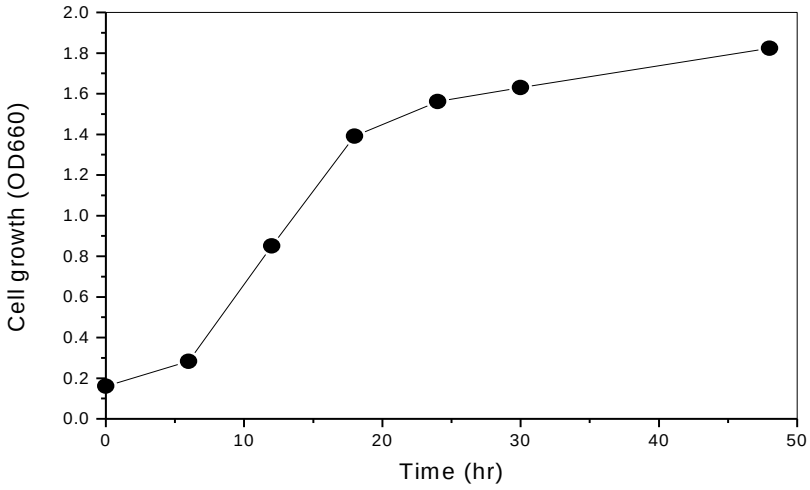

Fig. 7. Growth of *Erwinia carotovora* subsp. *carotovora.* Cells were grown at 40ºC for 48 hr in YG broth (pH 9.0)

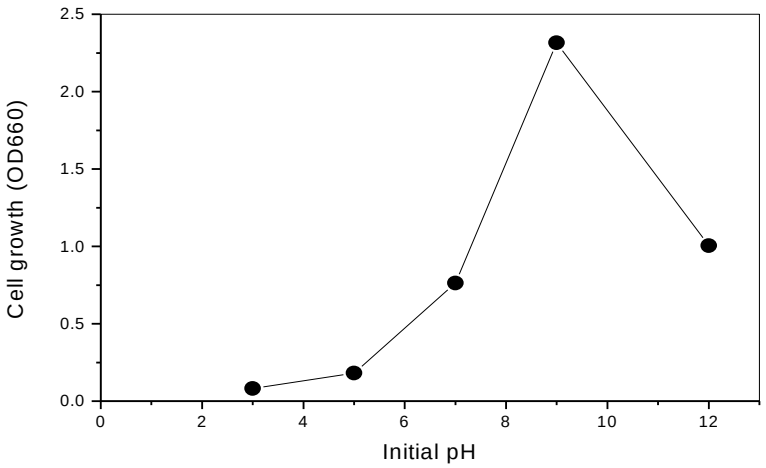

Fig. 8. Optimal initial pH for cell growth of Erwinia carotovora subsp. carotovora. Cells were grown at 40ºC for 24 hr in YG broth.

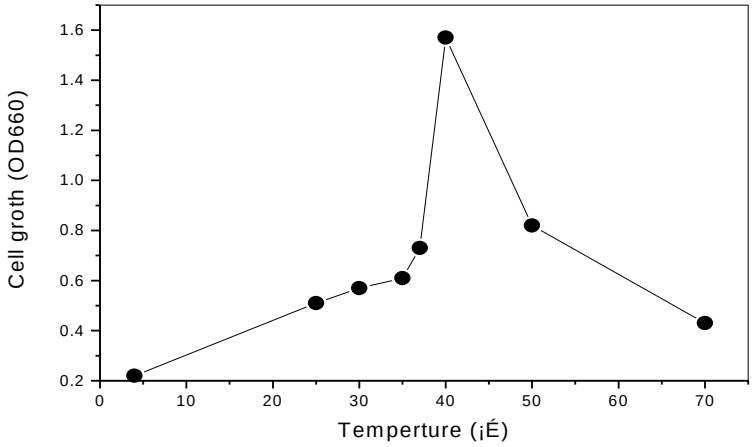

Fig. 9. Optimal temperature for cell growth of *Erwinia carotovora* subsp. *carotovora.* Cells were grown for 24 hr in YG broth (pH 9.0).

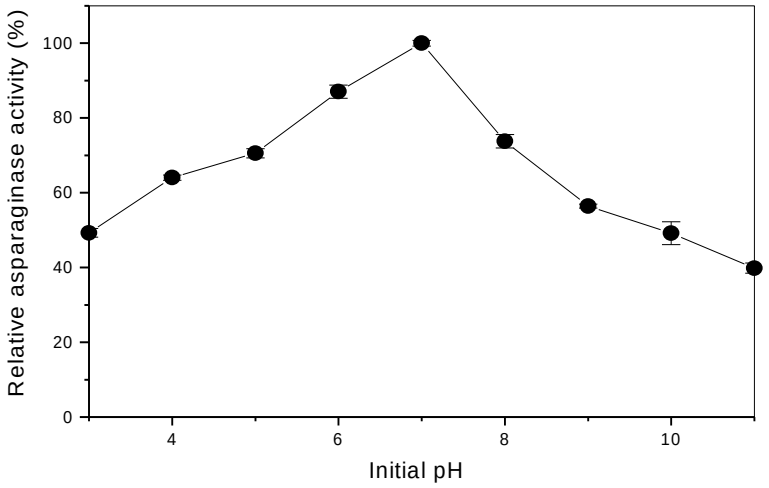

Fig. 10. Effect of initial pH for extracellular asparaginase activity. Cells were grown at 40ºC for 24 hr in YG broth.

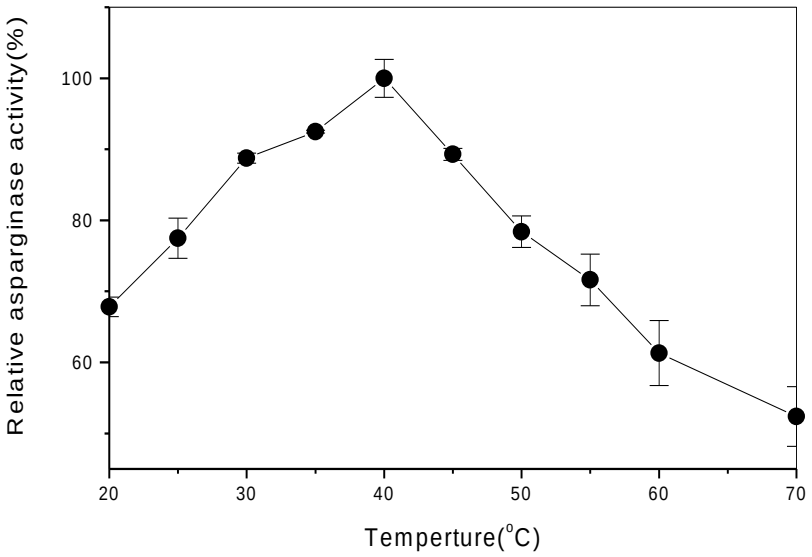

Fig. 11. Effect of temperature for extracellular asparaginase activity. Cells were grown for 24 hr in YG broth (pH 9).

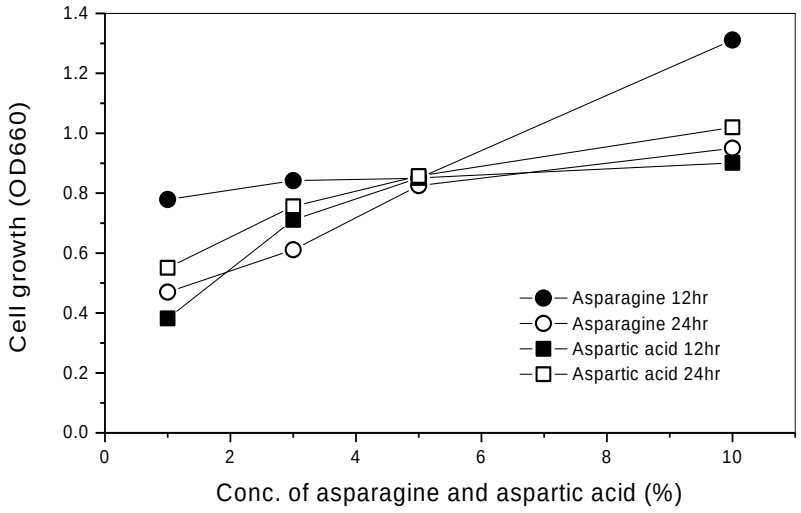

Fig. 12. Addition effect of asparagine and aspartic acid on cell growth of *E. carotovora* subsp. *carotovora.* Cells were grown at 40ºC for 24 hr YG broth (pH 9.0)

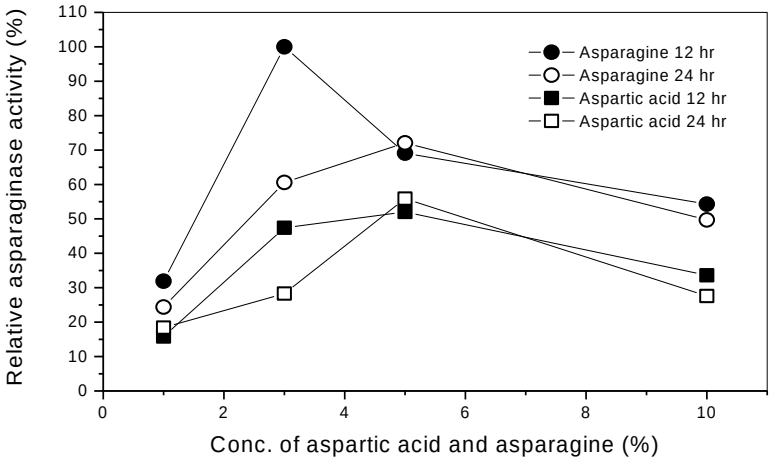

Fig. 13. Addition effect of asparagine and aspartic acid on extracellular asparaginase activity of *E. carotovora* subsp. *carotovora.* Cells were grown at 40ºC and in YG broth (pH 9) supplemented with asparagine and aspartic acid.

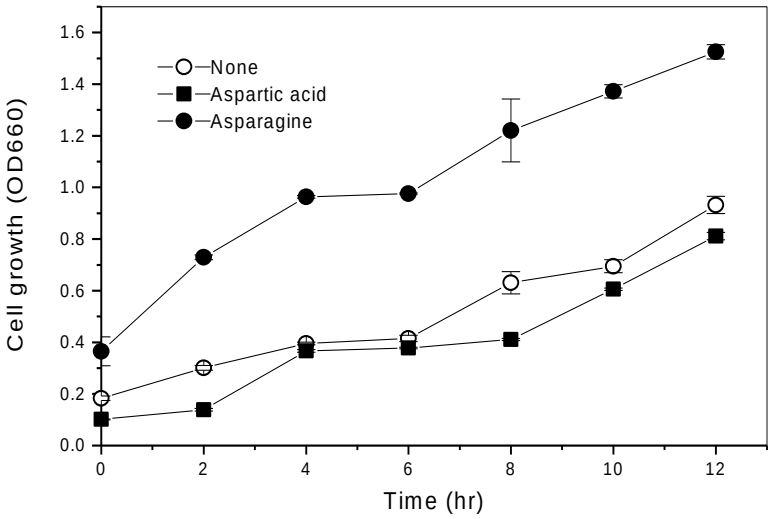

Fig. 14. Addition effects of asparagine and aspartic acid on cell growth of *E. carotovora* subsp. *carotovora.* Cells were grown at 40ºC and in YG broth (pH 9) supplemented with 3% of each asparagine and aspartic acid.

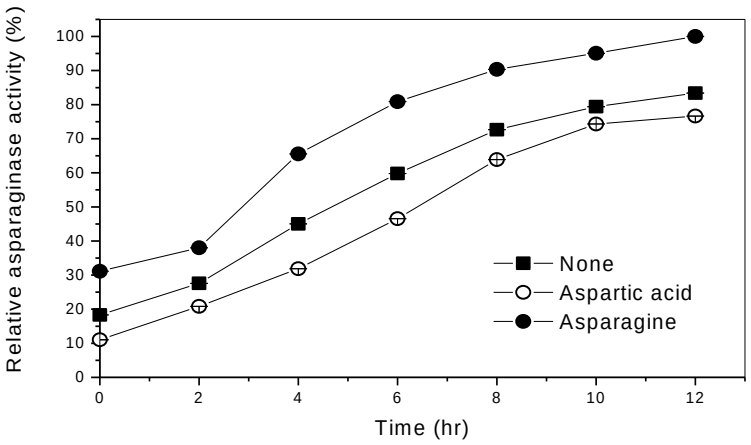

Fig. 15. Addition effects of asparagine and aspartic acid on asparaginase activity of *E. carotovora* subsp. *carotovora.* Cells were grown at 40ºC and in YG broth (pH 9) supplemented with 3% of each asparagine and aspartic acid.

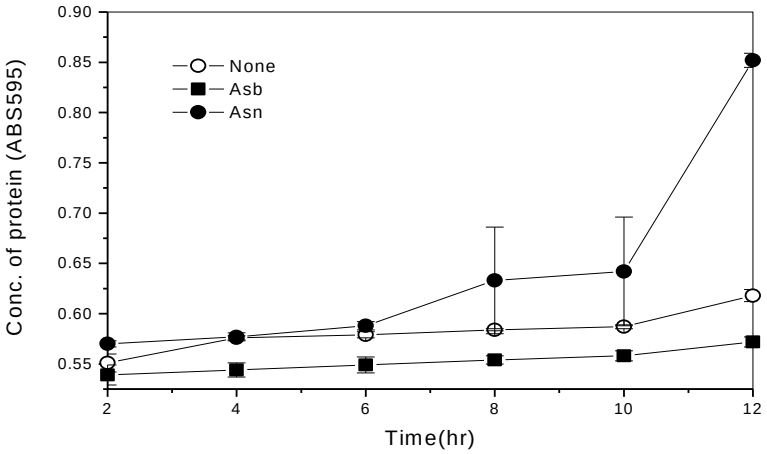

Fig. 16. Time course of extracellular protein production of *E. carotovora* subsp. *carotovora.* Cells were grown at 40ºC and in YG broth (pH 9) supplemented with 3% of each asparagine and aspartic acid.

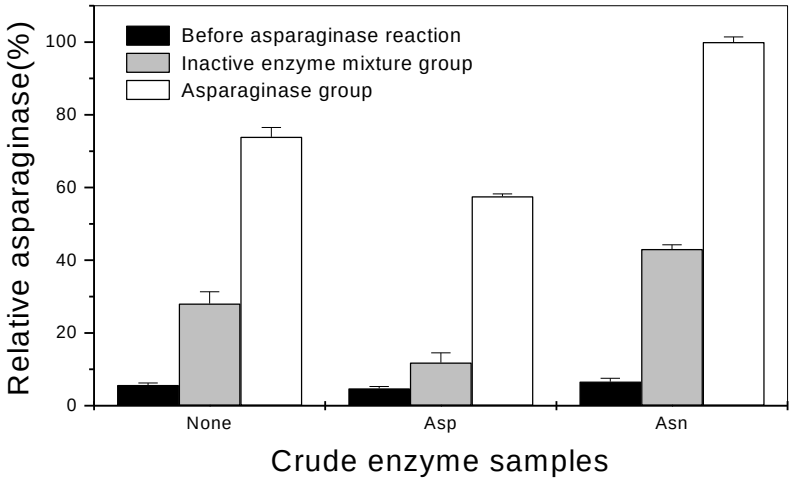

Fig. 17. Extracellular asparaginase activity using crude enzyme solution of *Erwinia carotovora* subsp. *carotovora*. Inactivated crude enzyme was prepared with hot water (100℃) treatment for 30 min.

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