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Optimization of Carbon-Nitrogen Ratio for Production of Gibberellic Acid by *Pseudomonas* sp.

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Abstract

In this study, favorable carbon-nitrogen ratio for high yields of gibberellic acid (GA_3) production from *Pseudomonas* sp. was investigated. First of all, optimum carbon (glucose, maltose, sucrose, fructose, lactose) and nitrogen (KNO_3, NH_4Cl, $NaNO_3$, urea, glycine) sources among the others were chosen. The highest yield of GA_3 productivity was found in growth medium supplemented with fructose (168.5 mg/L). $NaNO_3$ was found as a suitable nitrogen source (141 mg/L). Then, in order to determine the optimum carbon-nitrogen ratio, different concentrations of carbon (from 50 mM to 150 mM) and nitrogen (from 17 mM to 47 mM) sources were added in culture media. As a result, optimum carbon-nitrogen ratio for GA_3 production from *Pseudomonas* sp. was found to be 100:17 mM.

Key words: Plant growth hormones, gibberellic acid, carbon-nitrogen ratio

Introduction

Plant growth regulators such as gibberellins and cytokinins are economically and industrially important products. They are used commonly in agriculture, viticulture, gardens and horticulture (Bandelier and Renaud, 1997).

Gibberellins (GAs) are present naturally in plants in which they act as growth regulators. They are typical secondary metabolites in microorganisms. Upon exhaustion of nitrogen sources, exponential microorganismal growth ceases and secondary metabolism is triggered (Gelmi and Perez, 2000). More than ninety different types of gibberellins are known to occur in higher plants and microorganisms (Mander and Owen, 1996). Gibberellic acid (GA_3) is a commercially important phytohormone which regulates many different plant growth and development processes. In industrial scale, it is produced originally by submerged fermentation using *Gibberella fujikuroi*. Also, it can be obtained from several bacterial sources such as *Azotobacter*, *Azospirillium*, *Pseudomonas* (Rademacher, 1994; Basiacik, 1997). Gibberellic acid is an important biotechnological product. Especially, it is extensively used in agriculture, brewing and cosmetic industries. It has been reported that annual world production of gibberellic acid exceeds about 25 tons with a market value of 100 million USD (Tudzynski, 1999).

In this study, we used *Pseudomonas* sp. for GA_3 synthesis. We found convenient carbon-nitrogen ratio for increased GA_3 production. Carbon-nitrogen ratio is the most important factor in improving the yield of secondary metabolites in microorganisms (Elezar and Escamilla, 2000; Gelmi and Perez, 2000). In this work, we have determined the optimum carbon-nitrogen ratio for GA_3 production from *Pseudomonas* sp.

Experimental

Materials and Methods

Organism. Potent microorganism *Pseudomonas* sp. was isolated from wastes of Edremit Olive Oil factory. The culture maintained on nutrient agar (Difco) at +4°C in refrigerator with monthly transfer. Gibberellic acid was produced on synthetic nutrient broth (Difco) medium by *Pseudomonas* sp.

Inoculum and incubation. Culture stocks standardized by diluting with 0.9% NaCl until reaching Mc Farland 2 (approximately 6.3×10^6 cells/ml). Culture flasks (250 ml) were prepared containing 100 ml of nutrient broth medium. Growth media were

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sterilized at 121°C, 15 min., 1.5 atm by autoclave. *Pseudomonas* sp. was added to growth media (1 ml : 100 ml). The inoculated flasks were incubated at 30 ± 1°C for 3 days under dark conditions on a rotary shaker at 150 cycle/min. After incubation, bacterial growth was determined in nutrient broth by measuring turbidity at 450 nm.

Extraction of gibberellic acid. Culture media were filtered, then the pH of supernatants were adjusted to 2.5 with 37% HCl. They were extracted using liquid-liquid (ethylacetate/$NaHCO_3$) extraction (Cho *et al.*, 1979). Gibberellic acid in ethylacetate phase was measured by UV spectrophotometer (Jenway 6105) at 254 nm (Brückner and Blecschmidt, 1991).

Effects of carbon sources on GA_3 synthesis. For the determination of suitable carbon sources in GA_3 synthesis, *Pseudomonas* sp. was grown in medium containing varying carbon sources such as glucose, sucrose, fructose, lactose and maltose. Each of these carbon sources was added to nutrient broth at the concentration of 100 mM. Culture flasks were incubated under the same growth conditions.

Effects of nitrogen sources on GA_3 synthesis. KNO_3, $NaNO_3$, NH_4Cl, urea and glycine were added to the nutrient broth as nitrogen sources. They were sterilized by membrane filter and the final concentrations of nitrogen sources were adjusted to 37 mM.

Determinations of carbon-nitrogen ratio in GA_3 production. For this test two experimental protocols were established. Firstly, one nitrogen source ($NaNO_3$) was added to nutrient broth between 17–47 mM concentrations range. The carbon source was kept at fixed concentration. In the second protocol, the amount of nitrogen was constant and the suitable carbon source (fructose) was added between the concentrations of 50–150 mM into the growth medium.

Results and Discussion

Effect of nitrogen sources. In some studies, different nitrogen sources such as KNO_3, NH_4Cl, NH_4NO_3 were tested for their effects on the production of GA_3 and cytokinin (Pharis and Jones, 1987; Nieto and Frankenbeger, 1989; Cihangir and Aksöz, 1993). In our work, different nitrogen sources were added to each culture medium at 37 mM concentration. The highest GA_3 synthesis was found with the addition of $NaNO_3$ to the culture medium (141.6 mg/L), and the lowest amount of GA_3 was obtained in the NH_4Cl added medium (0.56 mg/L) (Table I). Therefore, we conclude that the microorganism shifted to the secondary metabolism faster when $NaNO_3$ was used. In other words, $NaNO_3$ encourages stationary phase and starts GA_3 production.

Effects of carbon sources. For the determination of the most effective carbon source in gibberellic acid synthesis from *Pseudomonas* sp. some carbon sources such as glucose, maltose, sucrose, fructose and

Table I
Effects of different nitrogen sources on growth and gibberellic acid synthesis by *Pseudomonas* sp.*

Nitrogen Sources (mM)	Growth (OD)	GA_3 (mg/L)	GA_3 Yields (mg/unit cells)
KNO_3	1.360 ± 0.006	85.5 ± 0.9	3.07 ± 0.03
$NaNO_3$	0.846 ± 0.005	141.6 ± 0.9	8.18 ± 0.06
NH_4Cl	1.386 ± 0.006	0.56 ± 0.6	0.02 ± 0.005
Urea	0.763 ± 0.004	68.5 ± 0.9	4.3 ± 0.1
Glycine	1.765 ± 0.005	55.1 ± 0.9	1.5 ± 0.1
Control	1.210 ± 0.005	136.4 ± 0.8	7.8 ± 0.1

* Culture flasks were incubated at 30 ± 1°C for 3 days under dark conditions on a rotary shaker at 150 cycle/min. Values represent means ± SD of 3 replicate cultures.

Table II
Effects of different carbon sources on growth and gibberellic acid synthesis by *Pseudomonas* sp.

Carbon Sources (mM)	Growth (OD)	GA_3 (mg/L)	GA_3 Yields (mg/unit cells)
Glycose	1.755 ± 0.01	63.5± 1.1	1.76± 0.2
Lactose	1.185 ± 0.04	150.1 ± 1.2	6.2 ± 1.8
Sucrose	1.504 ± 0.006	56.5 ± 1.1	1.8 ± 0.06
Fructose	1.011 ± 0.003	168.5 ± 0.6	8.1 ± 0.04
Maltose	1.340 ± 0.006	147.7 ± 1.1	5.3 ± 0.05
Control	1.155 ± 0.01	134.2 ± 1.1	6.3 ± 0.1

* Culture flasks were incubated at 30±1°C for 3 days under dark conditions on a rotary shaker at 150 cycle/min. Values represent means ± SD of 3 replicate cultures.

lactose were added to nutrient broth. It was found that the addition of fructose to the medium, increased gibberellic acid synthesis up to 168.5 mg/L (Table II). By metabolizing fructose, *Pseudomonas* sp. swiftly switched to the stationary phase and began synthesizing gibberellic acid. When glucose and sucrose was added to the medium the amount of gibberellic acid was low. This result states that glucose and sucrose have been utilized for stimulating growth of microorganisms. According to this data it can be concluded that while glucose and sucrose are suitable carbon sources for bacterial reproduction, fructose is appropriate for gibberellic acid synthesis. In various studies dealing with GA_3 and cytokinin synthesis, varied carbon sources

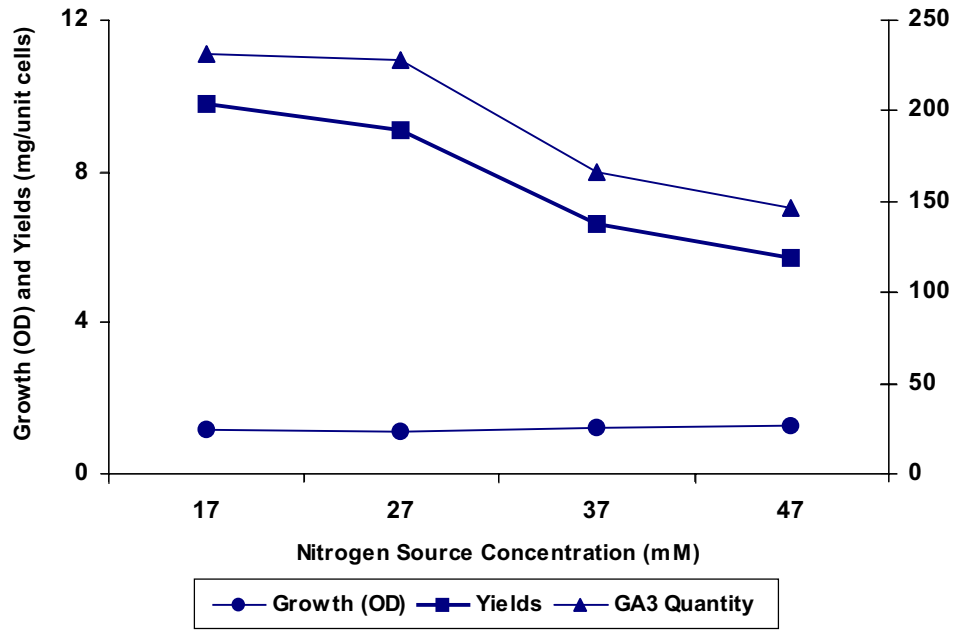

Fig. 1. Effects of different concentrations of $NaNO_3$ on growth and gibberellic acid synthesis.
Culture flasks were incubated at 30 ± 1°C for 3 days under dark conditions on a rotary shaker at 150 cycle/min.
Values represent means ± SD of 3 replicate cultures.

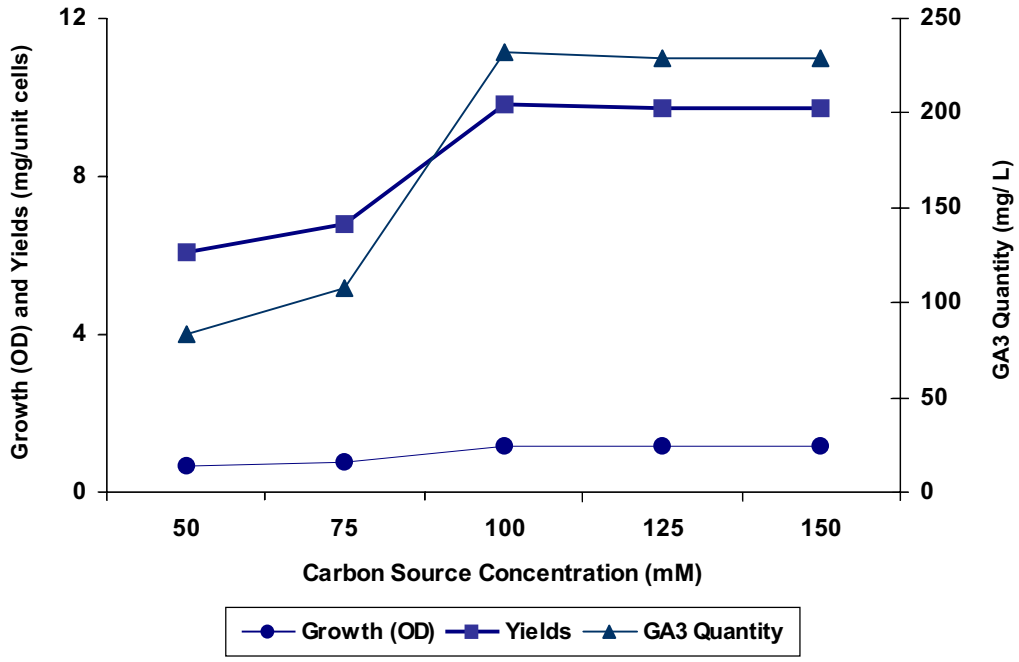

Figure 2. Effects of different concentrations of fructose on growth and gibberellic acid synthesis.
Culture flasks were incubated at 30 ± 1°C for 3 days under dark conditions on a rotary shaker at 150 cycle/min.
Values represent means ± SD of 3 replicate cultures.

such as sucrose and rafinose were found to be suitable for plant growth factor production (Lopez and Martinez,1988; Gulewicz *et al.*, 1994).

Carbon-nitrogen ratio. Determination of carbon nitrogen ratio is very important for the production of secondary metabolites in microorganisms (Cacciari *et al*., 1989). For this purpose we assembled mainly two experimental protocols for determination of a suitable carbon-nitrogen ratio in GA_3 production from *Pseudomonas* sp. As states before, in the first protocol, GA_3 value and yield was the highest in the medium with the lowest nitrogen source (17 mM) following incubation (Fig. 1). In the second protocol, nitrogen source was fixed and fructose as a carbon source was added between 50–150 mM concentrations range and the yield of GA_3 increased between these concentrations. The optimum carbon-nitrogen ratio was determined to be 100:17 mM (Fig. 2). GA_3 is a secondary metabolite and when nitrogen intake is limited then the secondary metabolism is accelerated. When nitrogen is limited, nitrogen is quickly used up for continuing bacterial growth and quantity of GA_3 increases (Gelmi and Perez, 2000; Eleazar and Escamilla, 2000). In conclusion, our results showed that the limitation of nitrogen in the medium and the use of the optimal concentration of a carbon source could increase the gibberellic acid production.

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