Korean J. Soil Sci. Fert. 43(4), 522-527 (2010) Article

Optimization of gibberellic acid production by *Methylobacterium oryzae* CBMB20

Md. Ashaduzzaman Siddikee, Muhammad Hamayun[1], Gwang-Hyun Han, and Tong-min Sa*

Dept. of Agricultural Chemistry, Chungbuk National University, Cheongju, Chungbuk, 361-763, Korea
[1]Dept. of Botany, Abdul Wali Khan University, Pakistan

Gibberellic acid (GA_3) is used in many industries and constitutes the primary gibberellins produced by fungi and bacteria. However, there is no information on GA_3 production by *Methylobacterium oryzae* CBMB20, a novel plant growth promoting bacterium. We investigated the favorable carbon (C) and nitrogen (N) sources and ratios and cultural conditions, such as incubation temperature, pH of the culture medium, and incubation period for the maximum production of GA_3 by *Methylobacterium oryzae* CBMB20. Maximum GA_3 production was observed in ammonium mineral salt (AMS) broth supplemented with Na-succinate and NH_4Cl as C and N sources, respectively. The maximum GA_3 production was found at the C/N ratio of 5:0.4 g L^{-1}. The highest GA_3 production was obtained when the bacterial culture was incubated at 30°C for 96 h at pH 7.

Key words: *Methylobacterium oryzae*, Gibberellic acid, Cultural parameter, Na-succinate, Ammonium chloride

Introduction

Soil bacteria generally known as plant growth promoting rhizobacteria (PGPR) can promote plant growth directly or indirectly via various mechanisms. Among the growth promoting mechanisms the production and release of stimulatory metabolites such as auxin, cytokinin (Timmusk et al., 1999 Madhaiyan et al., 2006a) and GAs (Joo et al., 2009 Kang et al., 2009) are very important. Gibberellins (GAs) are plant growth hormones which influence a wide range of developmental processes including stem elongation, seed germination and dormancy, sex expression and flowering and fruit sets and development in a range of plant species (Sponsel, 2003). Gibberellic acid is also an important biotechnological product and it is widely used in the cosmetic sector and beer industry. Although GAs are considered as typical secondary metabolites in microorganisms, until now their physiological roles are not known. Until recently, 136 GAs have been identified in 128 plant species, 28 GAs in 18 different fungi species and only four GAs (GA_1, GA_3, GA_4 and GA_{20}) in 12 different bacterial species (Hamayun et al., 2009).

Gibberellins have been unequivocally identified in cultures of *Azospirillum lipoferum* (Piccoli et al., 1996), *Azospirillum brasilense* (Janzen et al., 1992), *Acetobacter diazotrophicus* and *Herbaspirillum seropedicaea* (Bastian et al., 1998), *Acinetobacter calcoaceticus* (Kang et al., 2009) and *Burkholderia* sp. (Joo et al., 2009). Several observations led to suggest that these GA-producing microorganisms might induce or promote growth of the host plants by secreting these hormones in the rhizosphere (Bastian et al., 1998; Joo et al., 2009 Kang et al., 2009).

Methylobacterium is a group of strictly aerobic, facultative methylotrophic, Gram-negative and rod shaped bacteria able to grow on one-carbon compounds and a wide-range of multi-carbon growth substrates (Green and Bousifield, 1982). Moreover, *Methylobacterium* has a range of reported properties including nitrogen fixation (Jourand et al., 2004), IAA and cytokinin production (Omer et al., 2004), phosphorus solubilization, regulation of ethylene level in plant root by ACC deaminase (Madhaiyan et al., 2006a) and stimulation of resistance against pathogens (Madhaiyan et al., 2006b). However, GA production capacity for any member of the genus *Methylobacterium* has not been reported yet. In addition, production of GAs is considerably influenced by C and N sources, C/N ratio as well as other cultural conditions such as pH, temperature, and incubation time (Kahlon et al., 1986).

In this study, GA_3 production by the plant growth

Received : August 7. 2010 Accepted : August 13. 2010
*Corresponding author : Phone: +82432612561
E-mail: tomsa@chungbuk.ac.kr

promoting *Methylobacterium oryzae* CBMB20 and optimum culture conditions for a large scale production of GA_3 were investigated.

Materials and Methods

Bacterial strain and culture conditions *M. oryzae* CBMB20 was procured from the Plant Nutrition and Fertilizer Laboratory of the Department of Agricultural Chemistry, Chungbuk National University, South Korea. The culture was maintained on standard AMS medium at 30°C in incubator with monthly transfer. Various traits of *M. oryzae* CBMB20 are presented in Table 1. AMS broth was prepared and pH adjusted to 6.8-7.0 then 50 mL was transferred in 250 mL flasks to grow bacteria. Before inoculation of bacteria (1:50 mL), growth medium was autoclaved at 121°C for 15 min. The inoculated flasks were incubated under standard conditions for 3-5 days, and bacterial growth was monitored throughout the experiment by reading absorbance at 600 nm.

Extraction of GA_3 To extract and quantify the GA_3 produced by *Methylobacterium*, culture broth was centrifuged at 10,000 rpm for 15 min at 4°C. The pellet was discarded and the pH of the supernatant was adjusted to 2.5 with 2 N HCl. GA_3 were extracted using liquid-liquid (ethyl acetate/$NaHCO_3$) extraction method following Cho et al. (1979). GA_3 was extracted two times from the supernatant with ethyl acetate (Volume 1:10) and the upper ethyl acetate fraction that contains the free GA_3 was collected. To remove impurities, the ethyl acetate fraction was partitioned two times with 1 M $NaHCO_3$ (Volume 1:1) and the upper fractions were collected. The pH of the $NaHCO_3$ fraction was adjusted to 2.0. The $NaHCO_3$ solution with the free GA_3 was partitioned two times using ethyl acetate (Volume 1:10) and the upper fraction was collected. All the ethyl acetate fractions were composited together and dried using rotary evaporator and the residue was dissolved in 4 mL absolute methanol and filtered through Whatman No. 42 filter paper. The quantity of extracted GA_3 in absolute methanol was determined by comparing the absorbance of GA_3 dissolved in absolute methanol i.e. using standard curve of GA_3. The efficiency of recovery using this procedure was estimated to be 92% on the basis of samples in which 1 mg of GA_3 was added to AMS broth and extracted following the above described procedure.

Effects of C and N sources on growth and GA_3 production To determine the effects of different C and N sources on growth of *M. oryzae* CBMB20 and GA_3 production, *M. oryzae* CBMB20 was grown in the AMS broth

Table 1. Summary of plant growth promoting traits of *Methylobacterium oryzae* CBMB20.

Methylobacterium oryzae CBMB20		
Accession No. from DSMZ/KCTC, KACC collections		AY683047
Source of isolation		Rhizosphere of rice
Gram's reaction		-
Color		Pink
†IAA	Trp^+	+
	Trp^-	+
‡Cytokinin	t-ZR	+
	iPA	+
§ACC deaminase		+
¶ARA		+
§AHL		+

† IAA (Indole acetic acid) production with (Trp^+) or without (Trp^-) tryptophane supplementation.

‡ t-ZR (trans-Zeatin riboside), iPA (Iso pentenyl adenosine) are deferent form of cytokinin.

§ ACC (1-amino-cyclopropane-1-carboxylic acid) deaminase enzyme.

¶ ARA (Acetylene reduction activity) Ability to fixed atmospheric N_2.

§ AHL (Acyl-Homoserine Lactone) a quorum-sensing signaling molecule.

Ref: Madhaiyan et al. (2006a) and Poonguzhali et al. (2007).

supplemented with one of these C (Na-succinate, methanol, glucose, maltose, sucrose, fructose, and lactose) and N sources (KNO_3, NH_4Cl, $NaNO_3$ and glycine). Maximum GA_3 production was observed in the growth medium supplemented with Na-succinate and NH_4Cl. Each of these C and N sources was added to AMS broth at the concentration of 5 g L^{-1} for C and 0.2 to 1.0 g L^{-1} for N. Culture flasks were incubated under optimum growth conditions. After incubation, bacterial growth and the amount of GA_3 production were estimated following the above mentioned procedures.

Determination of C/N ratio for optimum growth and GA_3 production Two experiments were set to determine the optimum C/N ratio on bacterial growth and GA_3 production. In the first set, NH_4Cl was added to nutrient broth between 0.2 to 1.0 g L^{-1} concentrations range. The C source was kept at fixed concentration. In the second set, the concentration of N was kept constant and Na-succinate was added in the concentration range of 2.0 to 8.0 g L^{-1} into the growth medium.

Optimization of incubation period, temperature and pH for bacterial growth and GA_3 production To determine the optimum duration of incubation, temperature and pH, *M. oryzae* CBMB20 was inoculated into AMS broth, and the culture was incubated at different incubation period (12, 24, 36, 48, 72, 96 and 120 h at 30 ± 2°C), temperature (15, 20, 25, 30, 35 and 40°C for 96 h) and pH (6.0, 6.5, 6.8, 7.0 and 7.5) on a rotary shaker (150 rpm). After incubation, bacterial growth and the amount of GA_3 production were estimated following the above mentioned procedures.

Statistical analyses All data were subjected to analysis of variance and testing of means by Duncan's multiple range test (DMRT) at $P<0.01$ using the SAS package version 9.1. (SAS Institute Inc., Cary, NC, USA).

Results and Discussion

The effects of different C sources and different concentrations of Na-succinate on the growth and production of GA_3 by *M. oryzae* CBMB20 are presented in Fig. 1A and 2A. The addition of Na-succinate to the medium increased GA_3 production up to 124.5 mg L^{-1} and bacterial growth up to 1.49 OD_{600}, whereas the lowest bacterial growth and GA_3 production were found in the

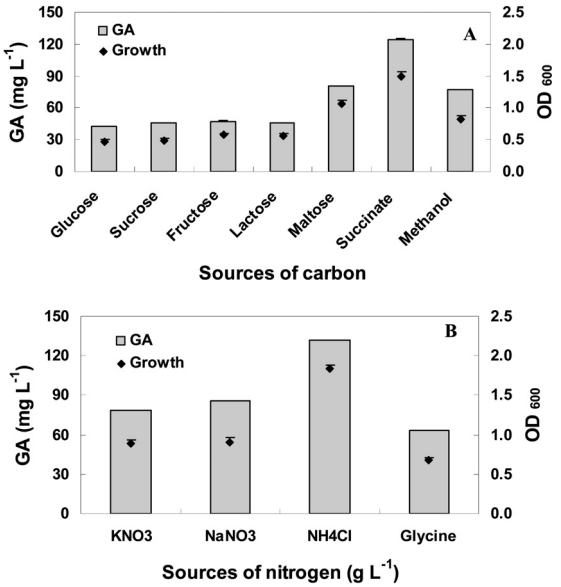

Fig. 1. Growth of *Methylobacterium oryzae* CBMB20 and GA_3 production in different carbon (A) and nitrogen (B) sources. OD_{600} stands for optical density at 600 nm.

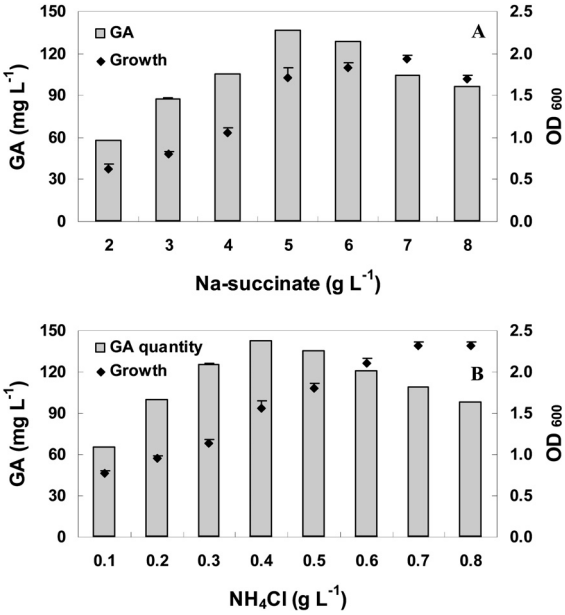

Fig. 2. Growth of *Methylobacterium oryzae* CBMB20 and GA_3 production at different concentrations of Na-succinate (A) and NH_4Cl (B). OD_{600} stands for optical density at 600 nm.

glucose and sucrose supplemented medium (Fig. 1A). Maltose and methanol on the other hand are considerable C sources only for bacterial reproduction. However, Na-succinate is appropriate both for bacterial growth and GA_3 production. Comparing the effect of the concentration of Na-succinate in the growth medium, the highest GA_3 production was found at 5 g L^{-1} but maximum bacterial growth was obtained at 7 g L^{-1} Na-succinate (Fig. 2A).

However, GA_3 production decreased when Na-succinate concentration was above 6 g L^{-1} and the bacterial growth also started to decrease. Bacterial growth increased with increased Na-succinate concentration but GA_3 production in the culture decreased. This reduced GA_3 production may be due to the higher amount of C which facilitate primary metabolism of bacteria and enhance growth and multiplication. Escamilla et al. (2000) also found that growth and production of GA_3 continues when sufficient C substrate is available for *Gibberella fujikuroi*. Demain (1998) reported that secondary metabolism is brought on by exhaustion of a nutrient, biosynthesis or addition of an inducer, and/or by a growth rate decrease. These events generate signals which affect a cascade of regulatory events resulting in chemical differentiation i.e., primary to secondary metabolism.

The effects of various N sources and their concentration on the bacterial growth and GA_3 production are presented in Fig. 1B and 2B. The highest bacterial growth and GA_3 production were found with the addition of NH_4Cl to the culture medium, and the lowest GA_3 production and bacterial growth were obtained in the glycine amended medium (Fig. 2B). Shukla et al. (2003) also found that N source plays a very significant role in gibberellic acid production. NH_4Cl was found to be suitable for maximum growth of *M. oryzae* CBMB20 and facilitate faster growth which rapidly shifted to the secondary metabolism. In other words, NH_4Cl encourages stationary phase and starts GA_3 production because it is a typical secondary metabolite in microorganisms. Upon exhaustion of N sources, exponential growth of microorganisms ceases and secondary metabolism is triggered (Escamilla et al., 2000).

Optimum concentration of NH_4Cl for GA_3 production was found to be 0.4 g L^{-1} whereas maximum bacterial growth was obtained at concentration of 0.7 g L^{-1} (Fig. 2B). Giordano et al. (1999) reported that in the biosynthesis of N-free compounds, GAs are inhibited by high amounts of N in the culture medium. The TOR kinase, responsible for regulating nutrient mediated growth signaling to control cell growth, has recently been implicated in the biosynthesis of GAs by *Fusarium fujikuroi* and was found to be involved in the N regulation of secondary metabolism and repression of the expression of GA biosynthetic gene in N sufficient conditions (Teichert et al., 2006).

To optimize C/N ratio for maximum bacterial growth and GA_3 production, two experiments were performed. The production of GA_3 was found highest in the AMS broth containing 5 g L^{-1} Na-succinate (Fig. 2A) and 0.4 g L^{-1} NH_4Cl (Fig. 2B). The limitation of N in the culture medium and the use of the optimal concentration of C source could increase the GA_3 production. Escamilla et al. (2000) reported that C/N ratio is the most important factor in improving the growth of microorganisms. Production of GAs starts when N is depleted and continues when sufficient C substrate is available for *Gibberella fujikuroi* (Escamilla et al., 2000).

The effects of different incubation periods, temperature and pH on growth and biosynthesis of GA_3 are presented in Fig. 3 (A, B and C). GA_3 production started to increase at 12 h of incubation and reached a maximum level after 94 h (Fig. 3A), and a decline of GA_3 production was observed at 120 h of incubation. Bacterial growth and GA_3 production persistently increased up to 72 h then growth stabilized but GA_3 production still increased. At this point, secondary metabolism may be triggered due to nutrient limitation. After 96 h of incubation, growth as well as GA_3 productions started to decrease rapidly. This is due to

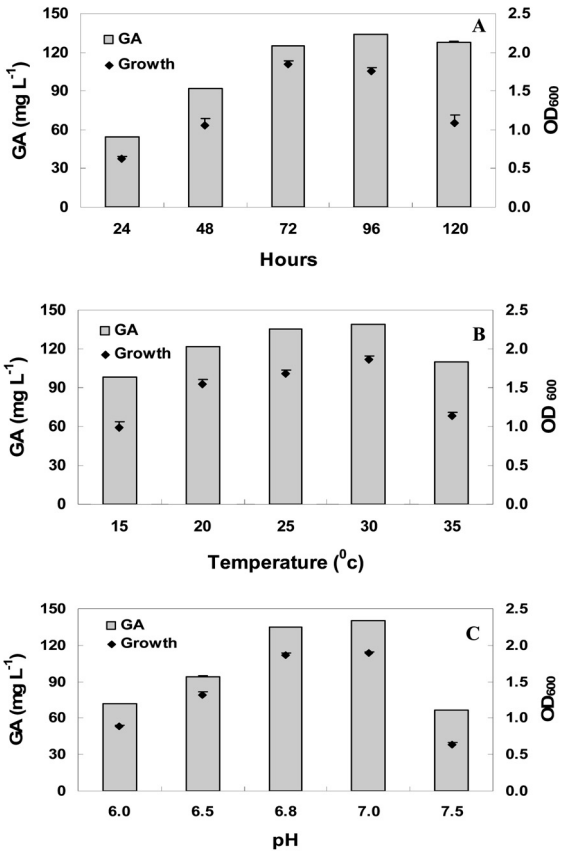

Fig. 3. Growth of *Methylobacterium oryzae* CBMB20 and GA_3 production at different incubation periods (A), temperatures (B) and pH (C). OD_{600} stands for optical density at 600 nm.

product inhibition, chemical decomposition and, perhaps, bio-degradation of the product by the culture (Hollmann et al., 1995). Hollmann et al. (1995) reported that GA_3 production stops and/or the concentration decreases sharply in *Gibberella fujikuroi* after 200 h. Similar to Escamilla et al. (2000) report on fungi, this studies showed maximum GA_3 production at the lag phase of growth.

Various incubation temperatures and pH showed a diverse effect on bacterial growth and GA_3 biosynthesis. Bacterial growth and GA_3 production increased with increasing temperature. The maximum GA_3 production and bacterial growth was detected at 30°C and both started to decrease at 35°C (Fig. 3B**).** Previous studies found the maximum GA_3 production at 30 ± 2°C (Zamanian et al., 1987). Escamilla et al. (2000) reported that temperature can affect the production of gibberellic acid in *Gibberella fujikuroi* and the activity of the GA_3 is lost at high temperature. The maximum bacterial growth and GA_3 production were obtained at pH 7.0, while lower GA_3 production and bacterial growth were obtained both at pH 6.0 and 7.5 (Fig. 3C).

All parameters studied in this study showed significant ($P \leq 0.01$) effects on the growth of *M. oryzae* CBMB20 and GA_3 production. Moreover, though each treatment showed less deviation from mean value of each parameter, value of CV confirmed that each treatment of all parameter has considerable impact on growth and GA_3 production (statistical inference has not shown).

In conclusion**,** GA_3 production by *M. oryzae* CBMB20 was highly dependent on the source of C and N, C/N ratio, and other cultural parameters. Based on the data obtained, the GA_3 production was maximum at 96 h of incubation at pH 7 at 30°C using Na-succinate and NH_4Cl as C and N source, respectively. Currently, GA_3 is largely produced by submerged fermentation of the fungus *Gibberella fujikuroi* (Santos et al., 2003) for 12 to 18 days of incubation. In addition, Kumar and Lonsane (1988) obtained GA_3 yield of 79 mg L^{-1} using non-conventional bio-reactor and immobilized mycelia of *G. fujikuroi*. In comparison to *G. fujikuroi*, *Methylobacterium* sp. may offer better options for GA production because it needs shorter incubation period and simple cultural conditions. These results suggest that *Methylobacterium* sp. can be used as a potential bacterial source to obtain high yields of GA_3. Moreover, *Methylobacterium* sp. could be a potential alternative of commercial GA_3 in agriculture by applying it as a plant growth promoting inoculant.

Acknowledgments

We thank Eleazar M. Escamilla S. for critical reading of this manuscript and giving constructive comments. This work was supported by the research grant of Chungbuk National University in 2010.

References

Bastian, F., A. Cohen, P. Piccoli, V. Luna, R. Baraldi, and R. Bottini. 1998. Production of indole-3-acetic acid and gibberellins A_1 and A_3 by *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* in chemically-defined culture media. Plant Growth Regul. 24:7-11.

Cho, K.Y., A. Sakurai, and Y. Kamiya. 1979. Effects of the new plant growth retardants of quaternary ammonium iodides on gibberellin biosynthesis in *Gibberella fujikuroi.* Plant Cell Physiol. 20:25-81.

Demain, A.L. 1998. Induction of microbial secondary metabolism. Int. J. Microbiol. 1:259-264.

Escamilla, E.M.S., L. Dendooven, I.P. Magana, R.S. Parra, and M. De la Torre. 2000. Optimization of gibberellic acid production by immobilized *Gibberella fujikuroi* mycelium in fluidized bioreactors. J. Biotechnol. 76;, 147-155.

Giordano, W., J. Avalos, O.E. Cerda, and C. Domenech. 1999. Nitrogen availability and production of bikaverin and gibberellins in *Gibberella fujikuroi*. FEMS Lett. 173:389-393.

Green, P.N., and I.J. Bousifield. 1982. A taxonomic study of some Gram-negative facultatively methylotrophic bacteria. J. Gen. Microbiol. 128:623-638.

Hamayun, M., A.K. Sumera, M.A. Khan, A.L. Khan, S.M. Kang, S.K. Kim, G.J. Joo, and I.J. Lee. 2009. Gibberellin production by pure cultures of a new of *Aspergillus fumigatus*. World J. Microbiol. Biotechnol. 25:1785-1792.

Hollmann, D., J. Switalski, S. Geipel, and U. Onken. 1995. Extractive fermentation of gibberellic acid by *Gibberella fujikuroi.* J. Ferment. Bioeng. 79:594-600.

Janzen, R., S. Rood, J. Dormar, and W. McGill. 1992. *Azospirillum brasilense* produces gibberellins in pure culture and chemically-medium and in co-culture on straw. Soil Biol. Biochem. 24:1061-1064.

Joo, G.J., S.M. Kang, M. Humayun, S.K. Kim, C.I. Na, D.H. Shin, and I.J. Lee. 2009. *Burkholderia* sp. KCTC 11096BP as newly isolated gibberellin producing bacterium. J. Microbiol. 47:167-171.

Jourand, P., E. Giraud, G. Bena, A. Sy, A. Willems, M. Gillis, B. Dreyfus, and P. de Lajudie. 2004. *Methylobacterium nodulans* sp. nov., for a group of aerobic, facultatively methylotrophic, legume root-nodule forming and nitrogen-fixing bacteria. Int. J. Syst. Evol. Microbiol. 54:2269-2273.

Kahlon, S.S. and S. Malhotra. 1986. Production of gibberellic acid by fungal mycelium immobilized in sodium alginate. Enzyme Microbe. Technol. 8:613-616.

Kang, S.M., G.J. Joo, M. Hamayun, C.I. Na, D.H. Shin, H.Y. Kim, J.K. Hong, and I.J. Lee. 2009. Gibberellin production and phosphate solubilization by newly isolated of *Acinetobacter calcoaceticus* and its effect on plant growth. Biotechnol Lett. 31:277-281.

Kumar, P.K.R. and B.K. Lonsane. 1988. Immobilized growing cells of *Gibberella fujikuroi* P-3 for production of gibberellic acid and pigment in batch and semi-continuous cultures. Appl. Microbiol. Biotechnol. 28:537-542.

Madhaiyan, M., S. Poonguzhali, J.H. Ryu, and T.M. Sa. 2006a. Regulation of ethylene levels in canola (*Brassica campestris)* by 1-aminocyclopropane-1-carboxylate deaminase-containing *Methylobacterium fujisawaense*. Planta 224: 268-278.

Madhaiyan, M., B.V.S. Reddy, R. Anandham, M. Senthilkumar, S. Poonguzhali, S.P. Sundaram, and T.M. Sa. 2006b. Plant growth promoting *Methylobacterium* sp. induces defense responses in groundnut (*Arachis hypogaea L.*) compared to rot pathogens. Curr. Microbiol. 53:270-276.

Omer, Z.S., R. Tombolini, A. Broberg, and B. Gerhardson. 2004. Indole-3-acetic acid production by pink-pigmented facultative methylotrophic bacteria. Plant Growth Regul. 43:93-96.

Piccoli, P., O. Masciarelli, and R. Bottini. 1996. Metabolism of 17, 17 [$^2H_2$]-Gibberellins A_4, A_9, and A_{20} by *Azospirillum lipoferum* in chemically-defined culture medium. Symbiosis 21:167-178.

Poonguzhali, S., M. Madhaiyan, and T.M. Sa. 2007. Production of Acyl-Homoserine Lactone Quorum-sensingsignals is wide-spread in Gram-Negative *Methylobacterium*. J. Microbiol. Biotechnol. 17:226-233.

Santos, M.G.E., M.C.M. Couto, and H.L.S. Rebelo. 2003. Ion-selective electrodes based on metalloporphyrins for gibberellic acid determination in agricultural products. Anal. Bioanal. Chem. 375:511-516.

Shukla, R., A.K. Srivastava, and S. Chand. 2003. Bioprocess strategies and recovery processes in gibberellic acid fermentation. Biotechnol. Biproc. Eng. 8:269-278.

Sponsel, V.M. 2003. Gibberellins. p. 29-40. In H.L. Henry and A.W. Norman (eds.) Encyclopedia of Hormones, Vol. 2. Academic Press, London, UK.

Teichert, S., M. Wottawa, B. Schonig, and B. Tudzynski. 2006. Role of the *Fusarium fujikuroi* TOR kinase in nitrogen regulation and secondary metabolism. Eukaryotic Cell. 5:1807-819.

Timmusk, S., B. Nicander, U. Granhall, and E. Tillberg. 1999. Cytokinin production by *Paenibacillus polymyxa.* Soil Biol. Biochem. 31:1847-1852.

Zamanian, M. and R.J. Mason. 1987. Benzene dioxygenase in *P. putida*. Biochem. J. 244:611-616.

지베렐린산 생산을 위한 *Methylobacterium oryzae* CBMB20의 최적 배양조건 확립

Md. Ashaduzzaman Siddikee · Muhammad Hamayun[1] · 한광현 · 사동민*

충북대학교 농업생명환경대학 농화학과, [1]파키스탄 압둘왈리칸대학교 식물학과

지베렐린산은 곰팡이 및 세균으로부터 생산되는 주요 식물생장촉진물질이며 다양한 산업에서 이용되고 있다. 본 연구는 *Methylobacterium oryzae* CBMB20을 이용하여 GA_3를 생산하기 위해 탄소원 및 질소원을 선발하였으며, 선발된 탄소원 및 질소원의 최적 농도와 최적 비율을 조사하였다. 탄소원으로는 Na-succinate가 methanol, glucose, maltose, sucrose, fructose, lactose에 비해 가장 우수하였으며, 질소원으로는 NH_4Cl가 NO_3, $NaNO_3$, glycine 등에 비해 우수하였다. 배양액에서 Na-succinate와 NH_4Cl를 각각 5 및 0.4 g L^{-1} 농도 비율로 사용하였을 때 GA_3의 생산량이 가장 높았다. 또한 ammonium mineral salt 배지의 pH를 7로 유지하고 30℃의 조건으로 96시간 배양하였을 때 GA_3의 생산이 최대로 나타나는 것을 확인하였다.