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THE PRODUCTION OF GIBBERELLINS FROM DIFFERENT AGRICULTURAL BY-PRODUCTS AS AFFECTED BY DIFFERENT CARBON AND NITROGEN SOURCES

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ABSTRACT

Gibberellins production from the basal fermentation medium (synthetic liquid medium of glucose and salts) by the fungus *Gibberella fujikuroi* NRRL 2284 was compared with its production from sugar cane and sugar beet molasses. The maximum production in liquid medium was 484.4 mg/l after 5 days of the basal incubation at 29 °C in shaking incubator at 200 rpm. The maximum production of gibberellins in sugar cane molasses (200 mg/l) was obtained when the sugar cane molasses was diluted to 8% of total sugars. While in beet molasses it was 141.4 mg/l when the sugar beet molasses was diluted to 6% of total sugars. The molasses media were supplemented with different nitrogen sources (NH_4NO_3, NH_4Cl, $(NH_4)_2SO_4$, $NH_4H_2PO_4$, CH_3COONH_4, and corn steep liquor (CSL). The best nitrogen source was CSL with nitrogen concentration 0.1 g/l. The effect of both carbon and nitrogen sources on the kinds of gibberellins produced was investigated by HPLC. The types of gibberellins were affected quantitatively and qualitatively.

INTRODUCTION

Gibberellins (GAs) are terpenoid plant hormones which involved in the control of different physiological processes such as: regulation of shoot elongation in many crop plants, regulation of flowering in some biennials or phtoperiodic plants, fruit ripening and development, etc. (Pharis and King 1985). Gibberellins are a large family of natural compounds, which are presented at low concentration in plant tissues, where they reached about 112 compounds (Tamotsu *et al.* 1998). The gibberellins were obtained industrially from the culture media of the fungus *G. fujikuroi*, which synthesizes about 20 different gibberellins, the most abundant of them is gibberellic acid (GA_3) (Rachew *et al.* 1993 and Martin *et al.* 1995).

Many researches were involved to produce the gibberellins by growing the fungus *G. fujikuroi* on different by-products as carbon sources, such as: whey (Gohlwar *et al.* 1984, Kahlon and Maihotra 1988; and Sastry *et al.* 1988); lupine seed extract (Gulewicz *et al.* 1994); sawdust of pine (Kukharskaya 1990); wheat bran (Kumar and Lonsane 1990, Qian *et al.* 1994, Kalra *et al.* 1995 and Bandelier *et al.* 1997); rice kernels (Latus *et al.* 1996); sugar cane bagasse (Tomasini *et al.* 1997); mussels processing wastes (Murado *et al.* 1993 and Pastrana *et al.* 1993) and manioc flour (Fang Weiming *et al.* 1999).

The aim of this work was to produce the fungal gibberellins from both sugar cane and sugar beet molasses as carbon sources, supplemented with different nitrogen sources, to explore the best one. The study also involve the

relation between both different carbon and nitrogen sources, in relation to different kinds of gibberellins produced by the fungus *G. fujikuroi* using HPLC technique.

MATERIALS AND METHODS

Agricultural by-products:

-Sugar cane molasses was obtained from El-Hawamdeia Factory for Sugar Industry. Sugar beet molasses was obtained from El-Delta Factory for Sugar Industry in Kafer El-Shikh. Corn steep liquor was obtained from Starch and Glucose Company in Mostorod.

The fungus:

The fungus *G. fujikuroi* NRRL 2284 was obtained from Northern Regional Research Laboratories, Illinois, U.S.A.

Microbial media:

Maintenance medium:

Potato Dextrose Agar (PDA) medium was used for the maintenance of the fungus, and for the inoculation of seed medium. It was prepared as follows: 200 g of peeled and sliced potatoes were boiled for 10 min in 500 ml of distilled water, then filtered through a gauze and the filtrate was restored to a volume of 500 ml. To this infusion were added (1) 100 ml of water contained 20 g dextrose, and (2) 400 ml of water contained 20 g of agar. The medium after dispersion was autoclaved for 20 minutes at 15 lb/in^2.

Seed medium:

This medium was used for the inoculation of fermentation and molasses media. It consists of glucose 20g; $(NH_4)_2SO_4$ 1g; KH_2PO_4 0.5g; $MgSO_4$ 0.5g. The complete medium was raised to 1litre with distilled water and the pH was adjusted to 4.5 (Tomasini *et al.*, 1997).

Basal fermentation medium:

This medium was used for the production of gibberellins. It has the following composition: Glucose 80 g; NH_4NO_3 0.48 g; KH_2PO_4 5 g; $MgSO_4.7H_2O$ 1g; and 2 ml of the following trace element solution: $FeSo_4.7H_2O$ 0.01g; $CuSO_4.5H_2O$ 0.015g; $ZnSO_4.7H_2O$ 0.161g; $MnSO_4.7H_2O$ 0.01g; Ammonium molybdate $(NH_4)_6Mo7O_{24}.4H_2O$ 0.01g ; distilled water to 100ml ; the complete medium was raised to 1litre with distilled water .The pH was adjusted to 4.5 (Geissman *et al.*, 1966).

Molasses media:

Serial dilutions of purified molasses (as will be mentioned in the methods) were made according to its content of total sugars from 1% to 10%. The pH was adjusted to 4.5.

Chemical analysis of agricultural by-products:

The chemical composition of sugar cane and sugar beet molasses, and corn steep liquor was determined according to the following:
The water and ash contents were determined as described by Leslie and Fisher (1971).

The spectrophotometric analysis of the agricultural by-products was used for the determination of total sugars (Dubois *et al.*, 1956); while GLC analysis (Hewlett Packard instrument) was used for the determination and identification of sugars (Kirrk and Sawyer, 1991).

The amino acids were determined by GLC as described by Gary (1990). The ammonium and nitrate nitrogen were determined by Kjeldahl method as adopted by Cottenie *et al.*, (1982).

Minerals (magnesium, ferrous, copper, manganese, zinc and molybdenum) were determined using Buck Scientific Atomic Absorption Instrument as described by Cottenie *et al.* (1982); while the sulfur and phosphorus were determined spectrophotometricaly using Beckman DU-600 spectrophotometer as described by Cottenie *et al.*, (1982).

Microbial production of gibberellins from the basal fermentation medium:

Seed medium:

Conical flasks (250 ml) containing 50 ml of this medium were sterilized at 121°C for 15min and inoculated with a mycelium grown for 5 days in PDA slant. These flasks were shaken in shaking incubator (200 rpm) for 48hr at 29°C (Tomasini *et al.*, 1997).

Fermentation medium:

The culture was performed in a 250 ml conical flask with 50 ml of fermentation medium. The medium was inoculated with 10% of seed culture and incubated for 14 days at 29°C in shaking incubator at 200 rpm. A sample was taken daily for gibberellins determination to determine the optimum time needed for the maximum production of gibberellins.

Microbial production of gibberellins from molasses media:

Purification of molasses:

The molasses was diluted 1:4 with acidified water (H_2SO_4 to pH 4.2-4.5), heated to 90°C for 30 min. and left over night. The formed precipitate was removed by filtration (Udeh and Achremowicz, 1994)

Molasses media:

Conical flasks (250 ml) containing 50 ml of the different dilutions of molasses media were sterilized at 121°C for 15min, inoculated with 10% of seed culture and incubated for 5 days at 29°C in shaking incubator at 200 rpm. The gibberellins were determined at the end of the incubation period to choose the optimum dilution of molasses media that give the maximum production of gibberellins.

Nitrogen sources:

Different nitrogen sources were added to the optimum dilution of molasses media, with the same concentration of nitrogen in the fermentation medium (0.168g nitrogen/l). The nitrogen sources were NH_4NO_3, NH_4Cl, $(NH_4)_2SO_4$, $NH_4H_2PO_4$, CH_3COONH_4, and corn steep liquor (CSL). The gibberellins were determined in each treatment at the end of the incubation period to choose the optimum nitrogen source that gives the maximum production of gibberellins.

Gibberellins analysis:

Extraction of gibberellins from the medium:

Ten ml of the medium was separated from the mycelia by centrifugation and brought to pH 8 with NaOH 1N. These were extracted three times with 5-ml ethyl acetate. The pH was brought to pH 2 with HCl 1N, and extracted three times with 5-ml ethyl acetate. (The pH was adjusted after every time of extraction, and the ethyl acetate was separated from the supernatant by centrifugation) (Martin *et al.*, 1995).

Determination of total gibberellins:

To 0.3 ml of the ethyl acetate acidic extract, one ml of 10 N HCl was added followed by 1 ml Folin Denis reagent and 3 ml H_2O, and mixed in a vortex. The test tubes were put in a boiling water bath for 5 minutes and left to cool at room temperature. The absorbance was measured at 750 nm using Beckman DU 600 spectrophotometer. A standard curve was prepared by 25 to 400 µg of gibberellic acid (Udagwa and Kinoshita, 1961).

Identification of gibberellins by HPLC:

One ml of the acidic ethyl acetate extract was evaporated under vacuum by rotary evaporator at 40°C, redissolved in 250 µl of methanol (HPLC grade), and 5 µl of this solution was injected into the Hewlett Packard 1100 HPLC instrument under the following condition: Hewlett Packard column ODS 200 mm x 4.6 mm x 5 µm; mobile phase: Methanol 30%, containing 0.01 M H_3PO_4 adjusted to pH 3 with KOH; flow rate 1 ml / min; variable wave length detector at 206 nm (Grolamys and Servando, 1997).

RESULT AND DISCUSSION

Chemical compositions of the agricultural by-products:

As illustrated in Table (1), sugar cane molasses contains greater amount of total sugars than sugar beet molasses, being 56.89% and 41.33% respectively. Sugar cane molasses also contain greater amount of sulfur than sugar beet molasses and CSL. It is 3.95, 1.02 and 2.05 respectively. Corn steep liquor contains the greatest amount of total nitrogen followed by sugar beet and sugar cane molasses, the values were 2.29%, 1.86% and 0.71% respectively. Most of this nitrogen is amino nitrogen, so that the analysis of the amino acids content was carried out. Corn steep liquor also contains the greatest amount of phosphorus followed by sugar cane and sugar beet

molasses, being1725 ppm, 26.6 ppm and 5 ppm, respectively. Sugar cane molasses contains greater amount of iron than sugar beet molasses and CSL, it is 133 ppm, 49 ppm and 11 ppm, respectively.

Table (1): * the chemical compositions of the agricultural by-products.

Chemical component	Agricultural by-product		
	Cane molasses	Beet molasses	C.S.L.
Water content %	26.7	18.7	60.2
Ash %	8.51	10.31	4.77
Total sugars %	56.89	41.33	2.94
Total nitrogen %	0.71	1.86	2.29
Amino nitrogen	0.6	1	1.58
Sulfur %	3.95	1.02	2.05
Mg %	0.33	0.04	0.11
P ppm	26.6	5	1725
Mn ppm	13	16	15
Cu ppm	10	6	4
Zn ppm	48	43	90
Fe ppm	133	49	110
Mo ppm	33.5	75.3	90

*The data was calculated by weight for molasses, and by volume for corn steep liquor

Sugars contents of the agricultural by-products:

Table (2) illustrates the sugars contents of the agricultural by-products. The major sugar in sugar cane molasses is sucrose (26.43g/100g), while it is glucose in sugar beet molasses (20.16g/100g), Both molasses contain the same sugars, with approximately the same amount of glucose, fructose and sorbitol.

Table (2):*GLC analysis of sugar in sugar cane and sugar beet molasses and corn steep liquor.

(Sugar)	Agricultural by-product		
	Cane molasses	Beet molasses	CSL
Glucose	18.07	20.16	0.4
Fructose	7.07	5.46	0.17
Sucrose	26.43	11.59	0
Galactose	0	0	0.12
Arabinose	0	0	0.26
Sorbitol	5.32	4.12	0.3

*The concentrations were calculated as g/100g for molasses, and g/100ml for CSL.

Amino acids contents of the agricultural by-products:

Table (3) illustrates that sugar cane molasses contain 17 amino acids. The most abundant of them is aspartic acid followed by cystein and alanine. Sugar beet molasses contains 18 amino acids where the most abundant of them is serine followed by methionine and aspartic acid, while CSL contains 17 amino acids the most abundant of them is aspartic acid followed by serine and proline.

Table (3): *GLC analysis of amino acids in sugar cane and sugar beet molasses, and corn step liquor.

Amino acid	Agricultural by-product		
	Cane molasses	Beet molasses	CSL
Alanine	39	107.6	63.5
Glycine	6.6	73.8	23.7
Threonine	1.17	14	9.9
Valine	19	57.7	1.5
Leucine + Isoleucine	2.67	49.9	59.3
Cystein	51.3	62.8	36.1
Serine	30.1	335.2	75
Aspartic acid	84.7	122.4	115
Proline	3.7	32.8	72.7
Hydroxy proline	1.49	6.3	1.6
Glutamic acid	5.5	53.9	26.8
Methionine	0	135.9	5.4
Arginine	24.1	0	70.4
Phenyl alanine	0.96	5.4	32.8
Lysine	0	9.4	65.8
Tyrosine + Histidine	2	80.9	34
Cystine	1.49	26.7	0

***The concentrations were calculated as mg/100g for molasses and mg/100ml for corn steep liquor.**

Effect of time on the production of gibberellins in fermentation medium:

As illustrated in Fig. (1), the maximum production of gibberellins reached after 5 days in the fermentation medium. This result is in agreement with the result obtained by Tomasini *et al.* (1997), when used two strains of *G. fujikuroi* NRRL 2278 and 5538. On the other hand the results (520 mg after 8 days) obtained by Dirk *et al.* (1995) when used a strain of *G. fujikuroi* DSM 893. The decrease in gibberellins concentration after 5 days is due to the decomposition of gibberellic acid, as mentioned by Dirk *et al.* (1995). The gibberellic acid is unstable in the aqueous phase under fermentation by *G. fujikuroi* NRRL 2284 in fermentation medium conditions, as it appears in pure GA_3 solutions in the absence of enzymes or microorganism. However, it may be increased by enzymes during the fermentation process. Gibberellinic acid (GE) is the stable product of GA_3 hydrolysis and has been found to be biologically inert. It may be detected in the medium after about four days after inoculation.

Effect of molasses dilution on the production of gibberellins:

As illustrated in Fig. (2), the maximum production of gibberellins reached to200mg/l in cane molasses diluted to 8% of its total sugars, this is the same sugar concentration in the fermentation medium, which is used in this study. While it reached to 141.4 mg /l in beet molasses diluted to 6%of its total sugars. This result is also in agreement with that obtained by Vanags *et al.* (1995) for the production of gibberellins by a strain of *G. fujikuroi* grown in a medium containing 6% raw sugar. There is constant production of gibberellins after 6% total sugars in sugar beet molasses. This is due to the

fact that the gibberellins production rate was inversely proportional to sugar concentration (Candau *et al.*, 1992). So that the production of gibberellins was delayed with the increase of carbon source concentration. On other hand, the gibberellins production decreased in cane molasses after 8% of total sugars concentration, while this was not happened in beet molasses; this effect may be due to the toxic effect of iron which is present in high concentration in cane molasses as shown in Table (1).

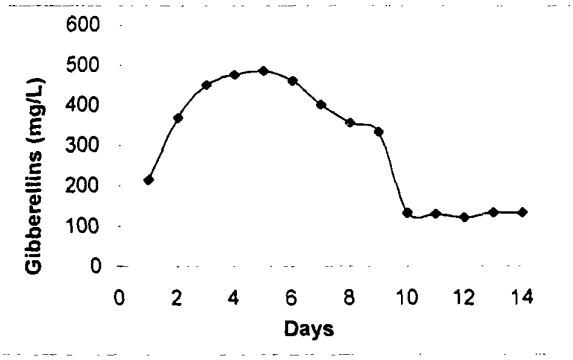


Fig. (1): The effect of time on gibberellins production by *G. fujikuroi* NRRL2284.

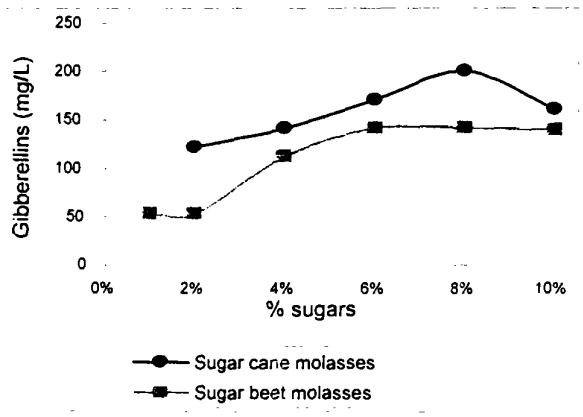
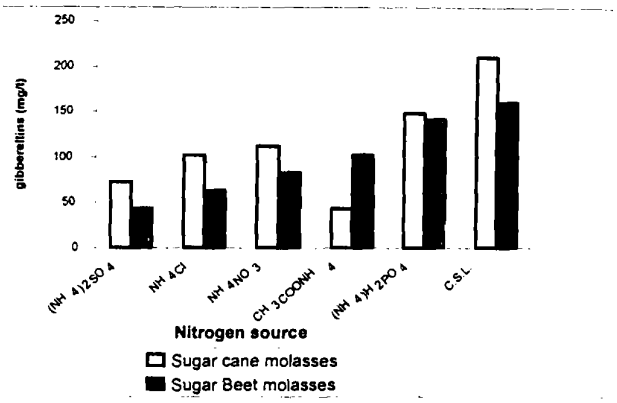


Fig. (2): The effect of molasses dilution on the production of gibberellins by *G. fujikuroi* NRRL2284.

The effect of different nitrogen sources on gibberellins production:

Figure (3) illustrates the effect of adding different nitrogen sources to molasses media on gibberellins production, as compared with ammonium nitrate which is used in the fermentation medium as a nitrogen source. The best nitrogen source for both sugarcane and sugar beet molasses was corn steep liquor. This is due to its content of free amino acids, and different precursors, which are important for fungus growth, in addition to vitamins

which acts as coenzymes for the enzymes involved in the biosynthesis of gibberellins. The gibberellins yield reached to 210 mg/l in cane molasses, and 160 mg/l in beet molasses. The lowest production obtained when ammonium acetate was used as nitrogen source in cane molasses medium, while in beet molasses it was ammonium sulfate. It is noted that the quantity of gibberellins produced in cane molasses is higher than that produced in beet molasses with all nitrogen sources, except with ammonium acetate.



Figure(3): The effect of different nitrogen sources on gibberellins production in cane and beet molasses by *G. fujikuroi* NRRL 2284.

Identification of gibberellins types produced in the fermentation medium:

The gibberellins were ordered according to their retention time from 1 to 28. In the fermentation medium there are 18 compounds, this result is in agreement with Robert (1972) who reported that this fungus synthesizes at least 16 gibberellins, and with Martine *et al.* (1995) who reported that this fungus synthesizes about 20 different gibberellins. The most abundant of these compounds is that have number 6-the gibberellic acid (Rt 6.8)- its quantity was 16.7% of total compounds. This result is in agreement with Rachev *et al.* (1993) and Martin *et al.* (1995). The compounds followed are have number 14 (14.8%); 8 (11.6%) and number 10 (10.1%).

Effect of different nitrogen sources addition on the types of gibberellins:

As illustrated in Fig. (5), there are qualitative and quantitative differences between the types of gibberellins produced in molasses media supplemented with different nitrogen sources. These differences may be due to the effect of the anion moiety on the activity of some enzymes, which involved in the biosynthesis of gibberellins.

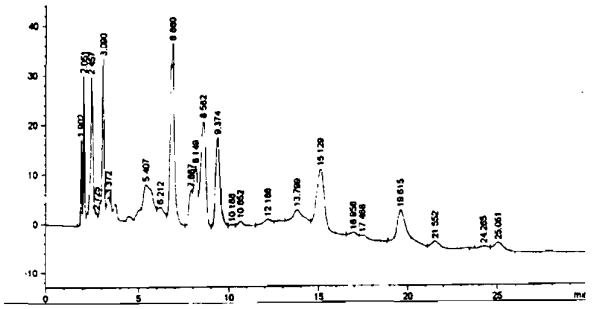


Fig.(4): HPLC chromatogram for the gibberellins produced in fermentation medium by *G. fujikuroi* NRRL2284.

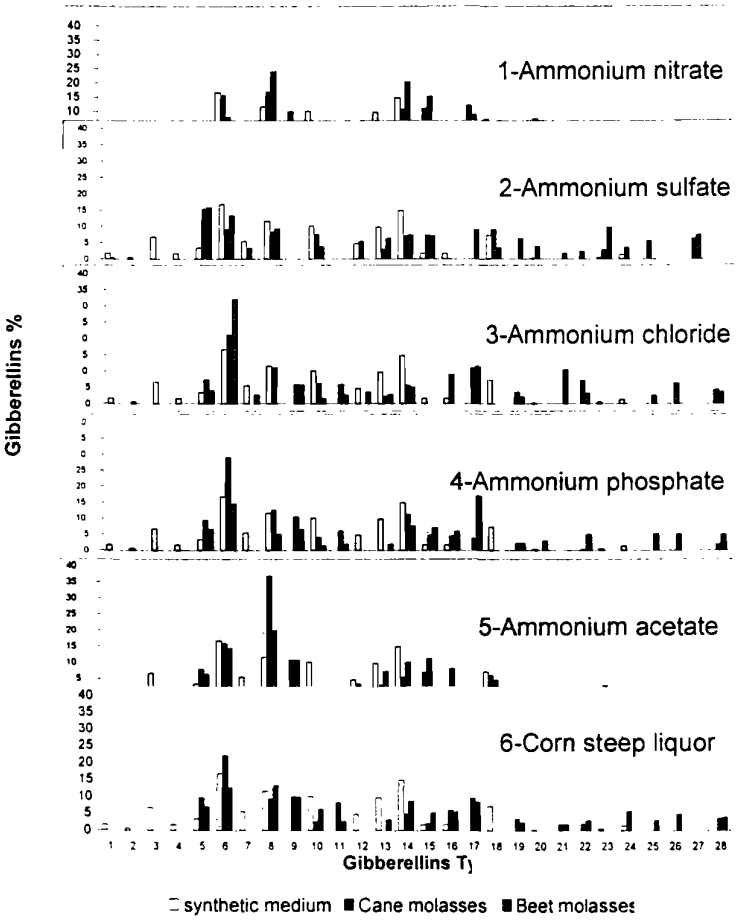


Fig. (5): Gibberellins types produced in molasses media as affected by different nitrogen sources, compared with fermentation medium.

It can be concluded for the production of gibberellins from sugar cane and sugar beet molasses, that the molasses is diluted to total sugars concentration 8% for sugar cane molasses and 6% for sugar beet molasses. Both of these media are supplemented with corn steep liquor with nitrogen concentration 0.1g/l and the pH is adjusted to 4.5. Both of them are inoculated with 10% of seed culture of the fungus *G. fujikuroi* NRRL 2284 and incubated at 29°C in a shaking incubator at 200 rpm for 5 days.

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إنتاج الجبريللينات من منتجات زراعية ثانوية مختلفة وأثر مصادر كربون ونيتروجين مختلفة

على رزق نصار-أحمد إبراهيم أبو شادى-نبيلة محمد رضوان*.

جامعة عين شمس - كلية الزراعة - قسم الكيمياء الحيوية الزراعية.

***مركز البحوث الزراعية - معهد بحوث الأراضي و المياه والبيئة - قسم الميكروبيولوجيا الزراعية.**

قورن إنتاج الجبريللينات في بيئة التخمر الأساسية(بيئة سائلة مخلقة من الجلوكوز و الأملاح المعدنية) بواسطة فطر *Gibberella fujikuroi* NRRL 2284 بالإنتاج من مولاس قصب السكر وبنجر السكر. أعلى إنتاج في البيئة السائلة الأساسية كان ٤٨٤,٤ مجم/لتر بعد خمسة أيام من التلقيح والتحضين على ٢٩° م فى محضن مع الرج على ٢٠٠ دورة في الدقيقة. أعلى إنتاج للجبريللينات في مولاس القصب (٢٠٠مجم/لتر) تم الحصول عليه عندما تم تخفيف المولاس إلى ٨% سكريات كلية. بينما فى مولاس البنجر كان ١٤١,٤مجم/لتر عندما تم تخفيف المولاس إلى ٦% سكريات كلية. تم إضافة مصادر نيتروجين مختلفة إلى بيئة المولاس NH_4NO_3, NH_4Cl, $(NH_4)_2SO_4$, $NH_4H_2PO_4$, CH_3COONH_4 وسائل نقيع الذرة(CSL). أفضل مصدر نيتروجين كان CSL بتركيز نيتروجين ٠,١جم/لتر. تأثير كلا من مصادر الكربون والنيتروجين المختلفة على أنواع الجبريللينات المنتجة تم دراسته باستخدام HPLC.أنواع الجبريللينات تأثرت كميا و نوعيا .