

Utilization of Some Sugar Industrial by- Products and Fructan Storing Crops for Bioproduction of Citric Acid

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ABSTRACT

Two sugar industrial by-products, cane bagasse “SCB” and sugar beet molasses “SBM” along with two fructan storing crops ,chicory roots“ CR” and Jerusalem artichoke tubers “JA”, alone or after mixing were utilized as a carbon source in fermentation medium to produce citric acid by *Aspergillus niger* strain GQ890276. It was found that the proximate composition and total sugars content of these agro-materials played a role in terms of citric acid production . Moisture ranged from 6.55 to 23.15%; crude fat from zero to 1.55%; crude protein from 3.27 to 8.53%; crude fiber from zero to 47.46% , ash from 3.29 to 8.93% ,nitrogen free extract from 44.83 to 87.06 % and total sugar 8.11 to 87.06% among these materials. The fermentation media either containing mixture of CR +SCB and/or CR as carbon source gave high citric acid yield after 7 days of fermentation comparing with other used carbon sources. The citric acid yield was 59.36 g/L in CR +SCB medium .It was 87.80% when calculation was based on the amount of consumed fermentable sugars. Also this media gave the high biomass (22.90 g/L) yield.

INTRODUCTION

Citric acid has a variety of uses in food, pharmaceuticals and industrial fields (Rohr, 1998). It's global production was more than one million ton per year (Papagianni, 2007). The fermentation production of citric acid is one of the large biotechnological industries. This process depends on using the filamentous fungus *Aspergillus niger* (Haq *et al.*, 2004 Demirel *et al.*, 2005). The economical production of citric acid by fermentation requires suitable inexpensive raw materials (Khosravi-Darani, and Zoghi, 2008). In the two last decades, a considerable interest has been focused on using agricultural products and their wastes for citric acid production by *Aspergillus niger* (Khosravi-Darani *et al.*, 2008). Bagasse and molasses are considered the main by-products of sugar industry. Bagasse represents nearly 30% of the sugar cane industry, meanwhile the produced molasses from using sugar beet in sugar production contains approximately 50% (w/w) total sugars (Leo, 1983). In other side, the discarded roots of chicory (*Cichorium intybus*) and Jerusalem artichoke tubers (*Helianthus tuberosus*, L.)

due to their collection at late maturation stage are rich in inulin ,a polymer of fructose with β 2-1 glycosidic linkage. (Gupta and Kaur, 1997).

Therefore, in the present study the above mentioned raw agro-materials, sugar cane bagasse and sugar beet molasses, chicory roots and Jerusalem artichoke tubers, alone or after mixing were evaluated as a carbon source in fermentation medium used for citric acid bioproduction using *Aspergillus niger* strain GQ890276 which was previously isolated from Egyptian dry sugarcane bagasse, identified as *A. niger* isolate MonEg by amplification and sequencing of its 18S rRNA (<http://www.ncbi.nlm.nih.gov/BLAST>).

MATERIALS AND METHODS

1. MATERIALS

Sugar cane bagasse (SCB) was obtained from Technological Lab. at Sabahia Agric. Research Station, Alexandria ,Egypt . It was first cut into small parts then dried in an air oven (E. Schulg & Co. Inh. Franz. KG) at 55°C for 12 hs and ground in a mill (Retsch GM200-Germany), sifted and kept in glass jars at room temperature (25±2°C).

Sugar beet molasses (SBM) was obtained from Delta Beet Sugar Company, Kafr El-Sheikh Governorate, Egypt.

The roots of chicory (CR) (*Cichorium intybus*) at late maturity stage were obtained from the experimental farm of Faculty of Agriculture, Alexandria University ,Alexandria , Egypt. The tubers of Jerusalem artichoke “J.A” (*Helianthus tuberosus*, L.) were obtained from Sabahia Horticultural Research Station Alexandria Egypt. The C.R and J.A samples were washed, sliced and rapidly immersed in 1% citric acid solution to avoid browning, then dried by hot air in a dryer at 55°C, to constant weight, grounded to pass through 60 mesh sieve and kept in dry glass containers until further uses.

Aspergillus niger strain GQ890276 was obtained from Technological lab. at Sabahia Agric. Res. Station, Alex. Egypt. It was previously isolated from Egyptian dry sugarcane bagasse. Its 18S rRNA was separated and purified according to instructions of Qiagen's DNeasy Kit (Qiagen, USA), amplified and its sequence were

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performed, according to the methods described by Guillemant and Drouard (1992). According to the obtained results of polymerase reaction (PCR) test and using Genbank database (<http://www.ncbi.nlm.nih.gov/BLAST>), this strain of this mold was submitted into genbank database under accession No.GQ890276. Figure (1) showed the phylogenetic tree of 18S rRNA of this isolated strain with 18S rRNA of the other strains of this mold from the data obtained from genbank database.

2. METHODS

Fermentation process: The fermentation medium for citric acid production consisted of the following compounds ((per liter of distilled water)) as described by Kirimura *et al* (1986): 1.6g; Yeast extract, 3 g; (NH₄)HPO₄, 0.5g; KCL, 0.5g; MgSO₄.7H₂O, 14mg; MnSO₄.7H₂O, 10 mg; FeCL₃.6H₂O, and 120g carbon source, from each of the following individual materials: sucrose, glucose, sugar beet molasses "SBM", sugarcane bagasse "SCB", chicory roots "CR", Jerusalem artichoke "JA", mixture of sucrose + SCB, molasses + SCB and CR +SCB at 1:1 w/w ratio. The pH of the medium was adjusted to 5.0 before distributing in flasks. The medium in the flasks were sterilized at 121°C for 15 min. then cooled to ambient temperature. The mold strain was inoculated on potato dextrose agar (PDA) slants and incubated at 30°C for 4-6 days. The spores of the mold were suspended in 8 ml of sterile-distilled water (Difco,1974) to use as inoculums. The sterilized flasks containing fermentation medium were inoculated with 10 ml of spore suspension containing about 1.7×10^8 spores/ml after adding 4% (v/w) methanol as a promoting substance for citric acid production (Khosravi-Darani, and Zoghi ,2008). The flasks were incubated at 30°C in a rotatory incubator shaker (Innova 4230,Edison, NJ., USA) for 7 days at 200 rpm (Roukas, and Liakopoulou-Kyriakides ,2002)

Extraction of citric acid: At the end of the incubation period, the mycelia and other suspended solids were removed from the fermentation media by filtration,

washed with 500 ml of distilled water, drying at 105 then weighted. After filtration, the filtrates were centrifuged at 3500g for 20 min at -4°C. Both citric acid and residual sugars were determined in the supernatant. Citric acid recovered from the supernatant by first precipitation as calcium citrate with calcium hydroxide then by acidification with sulphuric acid as following : the supernatant was first heated to about 60°C then calcium hydroxide was added until the neutralization point. The precipitated calcium citrate was removed by filtration and washed several times with distilled water to remove residual sugar, treated with 0.2% sulfuric acid and filtered to obtain the mother liquor containing citric acid (Ruijter and Visser ,1999).

Analytical Methods: Moisture content, total solids, crude fiber, ash, protein, total sugars, inulin and crude ether extract were determined according to AOAC (1998). Nitrogen free extract (NFE) was calculated by difference. Reducing sugars were analyzed by the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). Citric acid was determined by the colorimetric method of Marier and Boulet (1958). All experiments were conducted in triplicate.

Percentage of both yield of citric acid and utilized sugars during fermentation and volumetric productivity of citric were calculated using the following equations, (Lotfy *et a* , 2007):-

Yield of citric acid (%)= (grams of citric acid produced/ grams of original sugar) \times 100

Sugar utilization (%)= (grams of original sugar- grams of residual sugar / grams of original sugar) \times 100

Volumetric productivity of citric acid = g citric acid produced per liter per hour.

Statistical analysis: The obtain values of the produced citric acid from using the different carbon sources were subjected to statistical analysis using Co-Stat Software (2004) computer program for statistics. Student-Newman-Keuls test was used to compare means at (0.05) significance level.

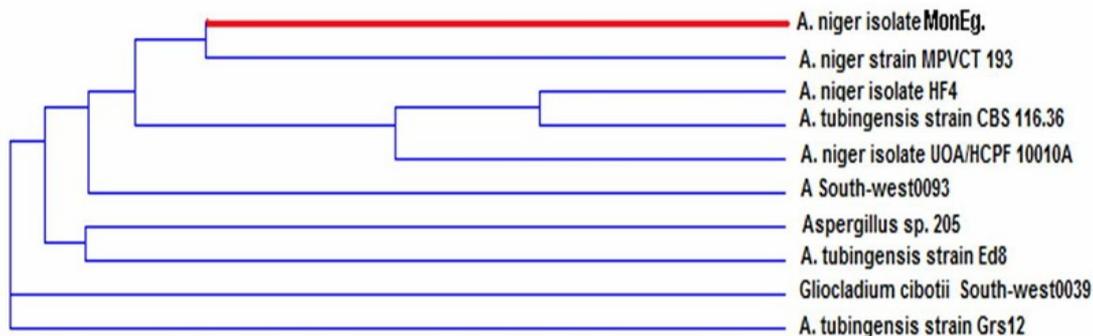


Figure1. Phylogenetic tree of 18S rRNA gene of MonEg. *A.nige*

RESULTS AND DISCUSSION

1-Proximate composition and sugars of the utilized agro-materials:

Data presented in Table (1) indicated a significant diversity in proximate composition and sugars of the utilized agro-materials. Moisture content varied from 6.55 to 23.15% among such raw materials. It was high in sugar beet molasses (SBM), low in sugar cane bagasse (SCB) and medium in both chicory roots (CR) and Jerusalem artichoke (JA) tubers. Crude protein content was high in JA (8.53%), low in SCB (3.27%) and nearly closed in SBM (4.01%) and CR (4.88%). Crude fat did not detect in SBM and was very low in SCB, CR and JA. The SBM had the highest ash content (8.93%), followed by JA (7.69%), CR (5.75%) and SCB (3.55%) respectively. SCB had the highest fiber content (47.46%) then CR (11.02%), and JA (4.46%). Meanwhile, SBM was free from this component. Nitrogen free extract was 87.06% in SBM, 78.06% in JA, 76.80% in CR and 44.83% in SCB. According to Beshay (2001) the bagasse of sugar cane contained 0.65 to 1.15% fat and 34.49 to 52% crude fiber. Monti *et al.*, (2005) found that crude protein and ash ranged from 8.56 to 15.73% and 9.58 to 13.75%, respectively in chicory roots. Also Praznik *et al.* (2002) and Amin, *et al.* (2005) reported that the lipid, ash and protein contents were $0.9 \pm 0.5\%$, 4.3 to 8.6%, and 6.6% respectively in dried tubers of Jerusalem artichoke. The percentage of total carbohydrate of sugar cane bagasse, and chicory pulp was 45.30% and 65.53%, respectively (Massoud, 2004).

As seen from data in table (1) total sugars was 8.11, 63.63, 12.45 and 21.38% in SCB, SBM, CR and JA,

Table. 1 Proximate composition, sugars and inulin of some agro- material (on dry weight basis)

Constituents %	Sugar cane bagasse (SCB)	Sugar beet molasses (SBM)	Chicory roots (CR)	Jerusalem artichoke tubers (JA)
Moisture	6.55 ± 0.99	23.15 ± 1.57	7.16 ± 1.09	9.14 ± 1.16
Crude protein (N×6.25)	3.27 ± 0.58	4.01 ± 0.22	4.88 ± 0.33	8.53 ± 0.15
Crude fat	0.89 ± 0.27	0.0	1.55 ± 0.25	1.26 ± 0.08
Ash	3.55 ± 1.05	8.93 ± 1.62	5.75 ± 0.81	7.69 ± 0.55
Crude fiber	47.46 ± 1.78	0.0	11.02 ± 0.92	4.46 ± 0.16
NFE*	44.83±3.68	87.06±1.84	76.8±2.31	78.06±0.94
Total sugar	8.11±0.52	63.63±0.73	12.45±0.80	21.38±0.61
Reducing sugar	2.05 ± 0.17	11.5 ± 1.30	1.96 ± 0.05	2.77 ± 0.08
Inulin	ND**	ND**	33.67 ± 0.72	16.55 ± 1.05

*NFE: Nitrogen free extract

**N D: Not detected

Results are mean ± SD of three determinations

respectively. Reducing sugars was lower (1.96%) in CR, SCB and JA comparing with SBM. The latter raw material contained at least 4 to 5 times of reducing sugars content found in the other materials. These values are nearly similar with those mentioned by Leo, (1983), El-Sharkawy, (1998), Femenia *et al.*, (1998) and Massoud *et al.* (2009) for the same materials.

Inulin was only detected in both CR (33.67%) and JA (16.55%), (Table 1). Such findings agree with the results of Patkai and Barta (2002). They reported that the inulin was 14-16.35% in JA.

2-Bioproduction of citric acid:

2.1-Effect of carbon sources: Results in Table (2) showed that the composition of the carbon sources in the fermentation medium influenced the yield of citric acid. Using disaccharides, sucrose, as carbon source gave higher yield of citric acid compared with monosaccharides, glucose. According to Kubicek and R?hr (1989) the extracellular mycelium of *A. niger* bound invertase which at low pH becomes active and rapidly hydrolyzes sucrose to glucose and fructose, 2 monosaccharides. Using sugar industrial by-products, sugar beet molasses and cane bagasse as carbon source in fermentation medium gave lower yield of citric acid comparing with using sucrose, (Table 2 and Fig.2). This was due to the low available sugars ($8.11 \pm 0.52\%$) in bagasse and high level of both ash ($8.93 \pm 1.62\%$) and total sugars ($63.63 \pm 0.73\%$) in molasses. The studies of Islam *et al.*, (1986); Kahlon, *et al.*, (1991) and Grewal and Kalra, (1995) indicated that the presence of metal ions in molasses reduced from the bioproduction of citric acid by *A. niger*.

Table 2. Effect of using some sugar-industrial byproducts and fructan storing plant as a carbon source on citric acid and consumed sugar and biomass yield*

Substrates	Citric acid (g/L)	Sugar utilization %	Citric acid yield (%)	Volumetric productivity of citric ^x	Biomass (g/L)	Citric acid (g/100g dry substrate)
Sucrose	24.99±1.17 ^{cd}	97.17±0.23 ^b	20.83±0.97 ^b	0.208 ^c	10.48±1.26 ^b	20.83
Glucose	21.42±2.09 ^{bc}	82.20±0.54 ^e	17.85±1.74 ^b	0.179 ^b	7.96±0.89 ^a	17.85
Sugarbeet molasses (SBM)	20.70±1.21 ^b	73.04±0.81 ^b	19.83±1.57 ^b	0.173 ^c	7.39±1.17 ^a	17.25
Sugarcane bagasse (SCB)	11.62±3.14 ^a	72.00±0.18 ^a	10.49±2.84 ^a	0.097 ^a	12.20±1.15 ^{bc}	9.68
Chicory roots (CR)	35.30±1.39 ^f	97.69±0.16 ^b	33.57±1.32 ^d	0.294 ^e	13.86±2.20 ^c	29.42
Jerusalem artichoke (JA)	26.01±2.37 ^d	92.75±0.12 ^a	26.27±2.39 ^c	0.217 ^c	14.05±1.75 ^{cd}	21.68
Sucrose + SCB(1:1)	31.50±1.57 ^e	80.50±0.35 ^d	27.31±0.94 ^c	0.263 ^d	16.45±1.09 ^{de}	26.25
SBM + SCB (1:1)	33.34±2.14 ^{ef}	77.54±0.88 ^c	31.00±1.98 ^d	0.278 ^{de}	17.37±0.96 ^e	27.78
CR+SCB (1:1)	59.36±1.21 ^g	87.80±0.26 ^f	55.01±1.13 ^e	0.495 ^f	22.90±1.72 ^f	43.63

Honecker *et al.*, (1989) found that the maximum yield of citric acid obtain when sugar content ranged from 14–22% in fermentation medium. Hossain *et al.* (1984) stated that adding carbon sources rich in total sugars in fermentation medium lead to suppress the activity of the enzymes produced by *A.niger* for the production of citric acid.

In the other side and as seen in Fig.(2) and Table (2) using each of fructan storing crops , chicory roots“ CR” and Jerusalem artichoke tubers “JA”, as a carbon sources in fermentation medium increased from the yield of citric acid than sucrose .Such increase may be attributed to the high level of inulin in both crops and especially in CR one . The latter crop had nearly the double amount of inulin in JA one ,Table(1). Results of Massoud *et al* (2010) showed that *A .niger* strain GQ890276 produced inulinase enzyme. Therefore it is able to hydrolysis inulin of CR and JA into fructose in fermentation medium and sequentially increase from yield of citric acid. Also CR and JA tubers may contain some activators such as minerals and vitamins which play role as a coenzymes during bioproduction of citric acid and increase from it's yield.

The above results were confirmed from the calculation of the consumed sugars, Table(2).The highest amount of consumed sugars was from the media containing CR followed by sucrose ,JA, glucose ,SBM and SCB, respectively .

Using 1:1 w/w mixtures of sucrose+SCB, SBM+SCB and CR + SCB as a carbon sources in citric acid fermentation medium caused a significance

increase in the acid yield. According to Kumar, *et al* (2003), sugarcane bagasse considered a suitable carrier in solid state fermentation method to produce citric acid by *A. niger*. Therefore it helps in increasing the yield of citric acid when used in previous mixtures.

The yield of citric acid in the fermentation medium containing CR + SCB mixture was more by nearly 40%than that containing the other two mixtures, Table (2) and Fig.(2).

Generally ,there was a parallel relationship between yield of citric acid as g/L and both percent of citric acid yield and as gram citric per 100 gram dry carbon source ,Table (2).

The highest production of biomass on dry weight was achieved when CR + SCB mixture was used as a carbon source followed by both SBM+SCB and sucrose +SCB, each of JA, CR, SCB then sucrose , both glucose, and SBM, respectively. This was due to differences in the growth rate of *A .niger* strain GQ890276 in the medium containing such different carbon sources.

2.2 Effect of fermentation time :This effect was studied in two fermentation mediums. The first contained SBM+SCB mixture and the second had CR+SCB mixture as a carbon sources.

As illustrated in Fig (3),a gradual increase in both citric acid and biomass yields was obtained with extending fermentation time to 7 days. A contrast trend was noticed for residual sugars. The rate of such changes

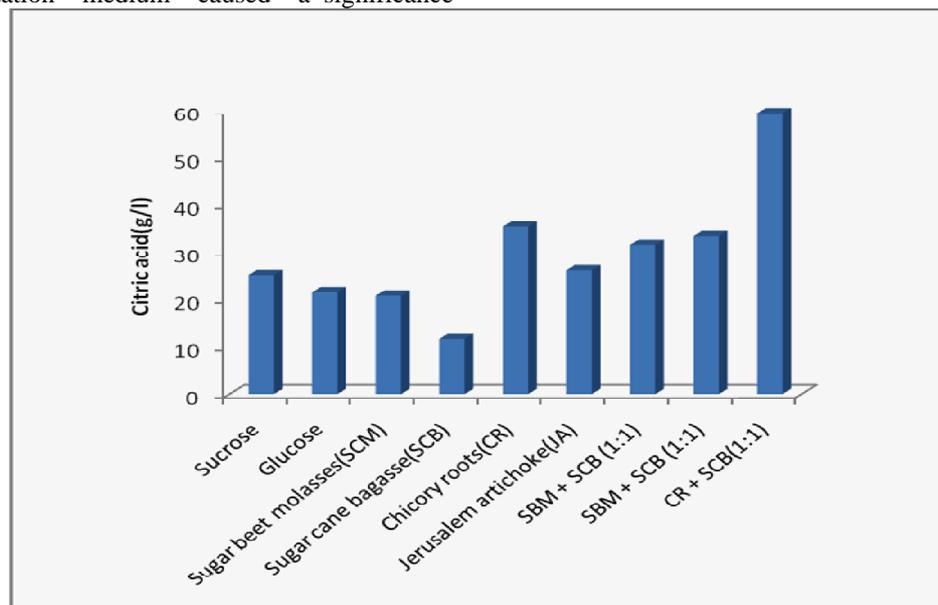


Figure 2. Effect of using different agro-materials as carbon sources on the bioproduction of citric acid yield

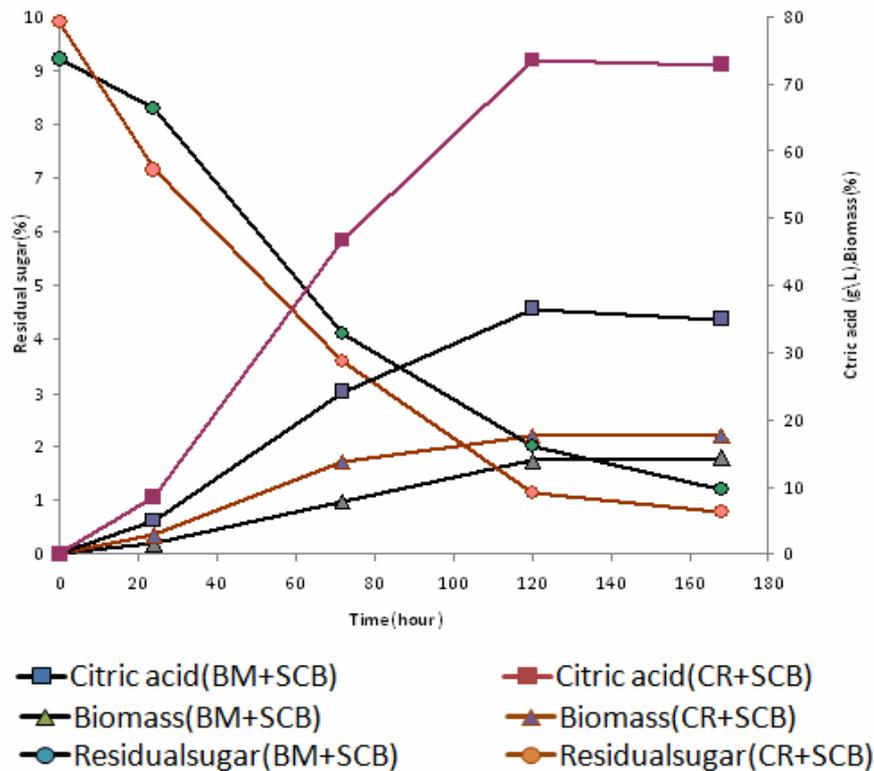


Figure 3. Effect of fermentation period on the yield of citric acid, biomass yield and residual sugar content

was differed according to both fermentation time and carbon source in medium. Generally a rapid acceleration rate for such changes was noticed through the first 5 days then turned to nearly stationary rate in the last 2 days of fermentation. As mentioned before, the yield of citric acid and biomass was higher and the residual sugar content was lower in fermentation medium containing CR + SCB than having SBM + SCB as a carbon source. Khosravi-Darani *et al* (2008) used pretreated and untreated straw as a carbon source to produce citric acid and found that the optimum fermentation time was 7 days. Kumar *et al* (2003) used fruit wastes for citric acid production using solid state fermentation process and found that maximum yield of citric acid was achieved after 9 days of fermentation.

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الملخص العربي

استخدام بعض مخلفات صناعة السكر والمحاصيل المخزنة للأنيولين للإنتاج الحيوي لحمض الستريك

منى ابراهيم مسعود، منى حسن نجيت

الى ٨٧,٠٦% والسكّريات الكلية من ٨,١١ - ٦٣,٦٣% بين هذه الخمات. أدى استخدام البيئة المحتوية على خليط بنسبة ١:١ من جذور نبات الشيكوريا ومصاصة قصب السكر وأيضا المحتوية نبات الشيكوريا كمصدر كربوني الحصول على أعلى عائد من حامض الستريك والكتلة الحيوية وأقل نسبة متبقية من السكّريات بالمقارنة بالبيئات الغذائية التي تحتوى على الخمات الاخرى. فكان عائد حامض الستريك ٧٣,٤٥ جرام/لتر والنسبة المستهلكة من السكّريات ٨٧,٨٠% والكتلة الحيوية على أساس وزن جاف ٢٢,٩٠ جرام/لتر بعد سبعة أيام من عملية التخمر في البيئة المحتوية على خليط من جذور نبات الشيكوريا ومصاصة القصب.

تم في هذه الدراسة استخدام اثنين من مخلفات صناعة السكر (مصاصة قصب السكر "SCB" ومولاس بنجر السكر "SBM") ومحصولين من المحاصيل المخزنة للأنيولين وهما جذور نبات الشيكوريا "CR" ودرنات خرشوفة القدس او الطرطوفة "JA" كمصدر للكربون أثناء الإنتاج الحيوي لحمض الستريك باستخدام سلالة فطر *A. niger* (GQ890276) السابق عزلها من مصاصة القصب. وقد وجد ان التركيب الكيماوى لتلك الخمات يؤثر على انتاج حامض الستريك. فلقد اختلف المحتوى الرطوبى من ٦,٥٥ الى ٢٣,١٥%، والمستخلص الاثيرى من صفر الى ١,٥٥% والبروتين من ٣,٢٧ الى ٨,٥٣%، والألياف الخام من صفر الى ٤٧,٤٦%، والرماد من ٣,٢٩ الى ٨,٩٣% والكربوهيدرات من ٤٤,٨٣