WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 6.805
Volume 5, Issue 3, 723-733. Research Article ISSN 2277– 7105

COMPARATIVE STUDY OF CITRIC ACID PRODUCTION FROM PUNICA GRANATUM AND ITS PEEL WITH EFFECT OF ALCOHOL AS A STIMULANT

M. Sashi Prabha[1*] and G. Subba rangaiah[2]

[1]Department of Microbiology, Dr. Lankapalli Bullayya P.G. College and Research center, Visakhapatnam, Andhra Pradesh, India.

[2]Department of Microbiology, Andhra University, Visakhapatnam, Andhra Pradesh, India.

Article Received on
12 Jan 2016,

Revised on 02 Jan 2016,
Accepted on 22 Feb 2016

***Correspondence for Author**
Dr. M. Sashi Prabha
Department of Microbiology, Dr. Lankapalli Bullayya P.G. College and Research center, Visakhapatnam, Andhra Pradesh, India.

ABSTRACT

Citric acid is one of the most important bulk produced organic acids,it is ubiquitous in nature. Citric acid is a 2-hydroxy propane,2,3-tricarboxylic acid ($CH_2COOH.COH.COOH.CH_2COOH$) which was first isolated from lemon juice and was crystallized by Scheele in 1784. In the present study more focus was made on the economical production of citric acid from Punica granatum and its peel, which was in turn compared with the rate of citric acid produced from sucrose as a substrate. Aspergillus niger MTCC 281 is the choice of the organism for the present study. Pomogranate (Punica granatum) is a seasonal fruit and its peel will be dumped indiscriminately after using the edible portion, and this activity may lead to environmental pollution. This environmental waste was considered for the present study as a substrate for citric acid production. The rate of production from fruit and its peel was in turn compared with the citric acid rate of production from sucrose. In the second part of the work three different alcohols were used (methanol, ethanol and butanol) to check the inhibitory or the stimulatory action of alcohol on citric acid production, and was compared. All the three different alcohols were used at five different concentrations.

KEYWORDS: Citric acid, Punica granatum, stimulants, substrate, Alcohols.

INTRODUCTION

Citric acid is one of the most important bulk-produced organic acids.[1] It is non-toxic and easily oxidized in the human body.[2] Because of its high solubility and palatability, it can be

used industrially for food and pharmaceuticals. Approximately, 75.0% commercial use of this acid is for food and 12.0% for pharmaceutical industries.[3] Citric acid obtained through the microbial fermentation is considered synthetic while that of present in fruits is referred to as natural.[4] Citric acid can be produced by the fermentation of glucose with aid of Aspergillus niger as a major organism but other than this we can use bacteria and other fungi.[5][6][7] There are many uses of this citric acid which have placed greater stress on increasing the citric acid production and search for more efficient processes.[8] The worldwide demand for citric acid is about 6.0×10^5 tons per year.[9] Some 400,000 tons are produced per year largely by process involving Aspergillus niger.[10]

The main aim of the present study is to study the citric acid production on the economical grounds using Punica granatum and its waste as a substrate which are considered as a municipal waste, because of environmental concern regarding the disposal of solid wastes. In addition, this process may give the encouraging results in terms of citric acid production. A variety of solids have been reported as substrate for the citric acid bioproduction, including kiwifruit peel[11], apple pomace, grape pomace[12], Wheat bran[13], sugar cane baggage, concentrated liquor of pineapple waste.[14] Sweet potato[15][16] and carrot.[17][18] The specific fruit that was selected is Punica granatum (Pomegranate) and its waste. Aspergillus niger (MTCC281) was selected for the production of citric acid. Comparative study was done with the sucrose as a substrate to that of the fruit and its waste as a substrates. The present study also deals with effect of alcohols as stimulants on citric acid production using fruit and its waste, so that we can get maximum amount of citric acid even from fruit waste which is considered as municipal waste. Three different alcohols were selected, i.e., Methanol, ethanol and butanol at different concentrations to check at which concentration we are getting more rate of yeild.

MATERIALS AND METHODS

Materials used

Organism used

Aspergillus niger MTCC281**,** The growth medium for the organism is Czapek Yeast Extract Agar medium.Received from Microbial Type Culture Collection and Gene Bank.

Instruments

pH meter, Autoclave, Orbital shaking Incubator, Colorimeter, Water bath, Electronic weighing balance.

Substrates

Punica granatum (Pomegranate) and its waste.

Methods

The initial sugar concentration has been found to determine the amount of citric acid and other organic acids produced in the culture broth.[19,20] Normally strains of A.niger need a fairly higher initial sugar concentration (15-18%, w/v) in the medium.[21,22] The higher sugar concentrations lead to greater amount of residual sugars making the process uneconomical.[23] So, presence of sugar concentration in the production media plays a crucial role in the rate of citric acid production. So, in order to determine the amount of initial sugar present in the substrate Anthrone's method was used.

The anthrone method for the determination of carbohydrates

There are different techniques for the estimation of carbohydrates. However, these determinations are associated with numerous difficulties. In order to overcome the difficulties Morse (1947)[24] and Morris (1948)[25], have described the use of anthrone for the quantitative estimation of carbohydrates which is both quicker and more accurate and suites well for the determination of carbohydrates. To obtain this degree of accuracy, it was found necessary to heat the mixture of the carbohydrate sample and the anthrone reagent at 100 ^{0}C. for 5 to 10 min after mixing.

Anthrone reagent

Anthrone reagent is prepared by dissolving 2 g. Anthrone in 1 L of 95% sulphuric acid. This Reagent has to be prepared fresh daily and was between 4 to 8 h old. After this time gradual increase in color occurred. After which it should not be used and has to be discarded.[26] Using the above stated method the amount of carbohydrate present in the Punica granatum (Pomegranate) and its waste was determined, in order to do so, for the sample preparation the Jackfruit and its waste was collected separately and macerated, together with the expressed juice dried in a hot air oven at less than 60 ^{0}C. They were then pulverized and stored in dark bottles.[27,28] Aliquots of ½ to 2 g. Pulverized material were used for analysis and followed the Morris anthrone method. The amount of carbohydrate in the test sample was estimated from a standard curve.

CITRIC ACID PRODUCTION

Microorganismu used

The microorganism used was the Aspergillus niger MTCC 281, received from Microbial Type Culture Collection and Gene Bank.

Shake Flask Studies

The Aspergillus niger cultures were used for citric acid production by submerged fermentation in 250 ml Erlenmeyer flasks.

Preparation of conidial iInoculum

Conidial inoculum was used in the present study. The spores from 4-6 days old slant cultures of PDA Medium were used for the inoculation. Inoculation is carried out using spores of A. niger.

Preparation of Vegetative Inoculum

To 100 ml of sterile fermentation media in a 1 L conical flask, 1 ml of the A.niger conidial suspension (1.2×106 culture per ml) was usedfor inoculation. The flask was incubated at 30 ^{0}C in a rotary shaking incubator at 200 rpm for 24 h.

Fermentation Technique

Vegetative inoculums were transferred into the sterile fermentation medium at a level of 4.0% (v/v). The Incubation temperature was kept at 30 ^{0}C throughout the fermentation period of 144h. The shaking speed of the orbital shaker was adjusted to 160 rpm.The pH of fermentation medium was adjusted to 3.5 by 0.1 N NaOH/ HCl before autoclaving. After the incubation period the ingredients of the flasks were filtered and the filtrate was used for the estimation of citric acid produced and residual sugar content. The dry cell mass was also calculated.

Recovery

Partial citric acid recovery was accomplished by the precipitation method.[29] After fermentation was completed fermentation broth was filtered completely. The filtrate was boiled with equivalent amount of lime and tricalcium citrate, this involves precipitation method. The calcium citrate was filtered off and then treated with sulphuric acid (60-70%, v/v) to obtain citric acid and precipitate of calcium sulphate.

Effect of Different Alcohols at Various Concentrations

The effect of different alcohols such as methanol, ethanol and butanol at varying concentrations on citric acid fermentation by the strain Aspergillu niger MTCC281, using Punica granatum (Pomegranate) fruits and its peels as a carbohydrate substrate in shake flasks, was carried out.

The concentration of alcohols varied from 0.5 to 2.5 % (v/v) in each case, i.e., with fruit and its waste, the same was performed with the standard production medium and was compared.

RESULTS

The critical parameters for citric acid production by Aspergillus niger were defined empirically, include high carbohydrate concentration but should not be more that 15- 20%. So, in order to ful fill the requirement the concentration of carbohydrates in Pomegranate and its peel was estimated and calculated (Table 1). So, 15 g/100 ml concentration of each fruit and its peel were calculated and were used for the present study of citric acid production using Pomegranate & its waste.Table2 has shown the data regarding the production of citric acid with Aspergillus niger MTCC 281 using Pomegranate and its wastes in shake flask method. The amount of sugar consumed,dry cell mass and citric acid produced was estimated(Table 2). According to Table 2,

The amount of citric acid obtained with control is 52.96±0.56 g/l, using sucrose as a substrate, where as with Pomegranate and its waste the yield obtained is13.16±0.59 g/l (Table 2) and 8.48±0.29 g/l (Table 2) respectively. The rate of yield from Pomegranate and its waste were compared with that of the control yield. The effect of alcohols as stimulants at various concentrations were also tested, alcohols used were Methanol (Table 3), Ethanol (Table 4) and Butanol (Table 5). After using different concentration of different alcohols as stimulants on all the three substrates, i.e., sucrose, Pomegranate and its waste we got highest of 61.98±0.03 g/l (Table 3) of citric acid with sucrose as a substrate at 1.0% Methanol as a stimulant, for Pomegranate and its waste, the highest amount of citric acid obtained is 19.85±0.24 g/l and16.32±1.11 g/l, respectively (Tables 4 and 5). In all the three cases 1.0% Methanol is acting as a good stimulants in compared to that of Ethanol and Butanol and other concentrations of methanol. Even though the amount of citric acid obtained with Pomegranate 13.16±0.59 g/l (Table 4) and its peel 8.48±0.29 g/l (Table 5) is less than the citric acid obtained from sucrose 52.96±0.56 g/l as a substrate, but the amount produced from fruit and its peel were not negligible,which has enhanced after the addition of stimulants to

19.85±0.24g/l and 16.32±1.11 g/l,for Pomegranate and its waste respectively. The point to be noted here is that the Ethanol and Butanol were not acting as a stimulant, in turn it is decreasing and inhibiting the rate of production in both the cases, i.e., with fruit and its peel.

Table 1: Estimation of carbohydrates in Pomegranate and its peel

Sl.No.	Name of the sample	Vol. of sample[1] (ml)	Conc. of sample for 0.1 mg (µg)[2]	Conc. of sample for 100 gm (gm)	Vol. of Anthrone(ml)	O.D. 620 nm
1	Pomegranate	1	17.69	17.69	4	0.17
2	Pomegranate peel	1	4.16	4.16	4	0.04

Note:

1. 1ml of volume of the sample = 0.1 mg of dried powder of the fruit/ sample
2. Concentration of sample was determined from the standard graph

Table: 2 Using of A.niger MTCC281 for citric acid fermentation, using Pomegranate and its in shake flask*

Sl.No	Sample	Dry cell mass (g/l)	Sugar consumed (g/l)	Citric acid (g/l)
1	Sucrose (Control)	15.97±0.49	97.99±0.56	52.96±0.56
2	Pomegranate	8.87±0.04	80.63±0.34	13.16±0.59
3	Pomegranate peel	9.06±0.04	101.48±0.28	8.48±0.29

Note:

* Fermentation period 168 h, Sugar concentration 150 g/l, Initial pH 2.5, incubation temperature 30 ^{0}C.± Indicate standard error mean (SEM) of the mean.

Table:3 Effect of methanol, ethanol and butanol at various concentration on citric acid fermentation by the Aspergillus niger MTCC281 using Sucrose salt medium in shake flasks*

Sl.no	Sample	Alcohol	Concentration %	Dry cell mass(g/l)	Sugar consumed(g/l)	Citric acid(g/l)
1	Sucrose	-	-	15.97±0.49	97.99±0.56	52.96±0.56
2	Sucrose	Methanol	0.5	16.02±0.42	95.31±0.29	56.60±1.29
			1.0	15.69±0.50	96.74±0.07	61.98±0.03
			1.5	15.33±0.06	95.87±0.29	61.66±0.38
			2.0	14.92±0.53	94.92±0.38	57.79±0.39
			2.5	16.43±0.73	95.24±0.33	53.45±0.18
3	Sucrose	Ethanol	0.5	16.51±0.37	100.40±0.35	49.60±1.29
			1.0	16.93±0.26	101.44±0.74	53.98±0.03
			1.5	16.96±0.03	101.92±0.88	53.66±0.38
			2.0	16.48±0.51	102.70±1.31	50.79±0.39
			2.5	16.75±0.38	101.26±0.59	46.45±0.18
4	Sucrose	Butanol	0.5	13.98±0.39	101.29±0.25	38.93±0.57
			1.0	13.68±0.49	102.76±0.06	42.31±0.87

			1.5	13.35±0.06	101.86±0.28	39.66±0.38
			2.0	12.90±0.50	100.93±0.38	36.46±0.28
			2.5	14.42±0.70	101.26±0.33	32.79±0.31

* Initial sugar concentration 150g/l, Fermentation period of 168 h, incubation, 30 ^{0}C, and initial pH 2.5.

Each value is an average of three parallel replicates. ± Indicates standard error mean among the replicate.

Table 4: Effect of methanol, ethanol and butanol at various concentration on citric acid fermentation by the Aspergillus niger MTCC281 using Pome granate as a substrate in shake flasks*

Sl.no	Sample	Alcohol	Concentration %	Dry cell mass (g/l)	Sugar consumed (g/l)	Citric acid (g/l)
1	Pome granate-	-	-	8.87±0.04	80.63±0.34	13.16±0.59
2	Pome Granate	Methanol	0.5	8.08±0.46	96.58±0.59	16.88±0.43
			1.0	7.78±0.26	96.30±0.15	19.85±0.24
			1.5	8.53±0.19	96.68±0.74	17.80±0.28
			2.0	7.41±0.23	97.43±0.62	16.07±0.76
			2.5	8.12±0.25	98.22±0.40	14.01±0.83
3	Pome Granate	Ethanol	0.5	10.52±0.19	106.59±0.59	9.69±0.19
			1.0	9.52±0.36	106.01±0.47	12.59±0.08
			1.5	10.33±0.13	106.75±0.74	11.53±0.05
			2.0	9.51±0.23	107.49±0.64	8.47±0.13
			2.5	9.93±0.50	108.25±0.42	5.38±0.21
3	Pome Granate	Butanol	0.5	5.86±0.24	105.05±0.42	3.41±0.46
			1.0	5.54±0.27	105.18±0.40	7.14±0.34
			1.5	5.32±0.13	105.03±0.37	5.35±0.41
			2.0	5.81±0.37	104.61±0.23	1.46±0.13
			2.5	5.91±0.55	105.50±0.29	0.00

* Initial sugar concentration 150g/l, Fermentation period of 168 h, incubation, 30 ^{0}C, initial pH 2.5.

Each value is an average of three parallel replicates. ± Indicates standard error mean among the replicates.

Table 5: Effect of methanol, ethanol and butanol at various concentration on citric acid fermentation by the Aspergillus niger MTCC281 using Pome granate peel as a substrate in shake flasks*

Sl.no	Sample	Alcohol	Concentration %	Dry cell mass(g/l)	Sugar consumed (g/l)	Citric acid (g/l)
1	Pomegranate peel	-	-	9.06±0.04	101.48±0.28	8.48±0.29
2	Pomegranate peel	Methanol	0.5	7.18±0.19	78.29±0.60	10.71±0.17
			1.0	6.91±0.57	78.03±0.16	16.32±1.11
			1.5	7.32±0.22	78.11±0.86	14.23±0.52
			2.0	6.57±0.22	78.52±0.34	9.50±0.24
			2.5	6.95±0.25	78.91±0.42	8.44±0.17
3	Pomegranate peel	Ethanol	0.5	8.22±0.19	86.29±0.59	4.72±0.13
			1.0	7.88±0.55	86.04±0.14	7.63±0.24
			1.5	8.36±0.22	86.78±1.06	6.57±0.33
			2.0	7.55±0.18	87.53±0.34	4.84±0.32
			2.5	7.96±0.26	87.95±0.42	3.74±0.38
3	Pomegranate peel	Butanol	0.5	5.20±0.17	84.31±0.59	1.42±0.13
			1.0	5.25±0.36	84.03±0.16	5.18±0.07
			1.5	5.32±0.21	84.10±0.86	2.42±0.06
			2.0	5.25±0.53	84.53±0.35	1.50±0.23
			2.5	5.28±0.33	84.95±0.38	0.65±0.65

* Initial sugar concentration 150g/l, Fermentation period of 168 h, incubation, 30 ^{0}C, initial pH 2.5.

Each value is an average of three parallel replicates. ± Indicates standard error mean among the replicates.

DISCUSSION

Citric acid produced from Pomegranate and its waste were compared with sucrose as a substrate for citric acid production (Table 2). In order to increase the yield, alcohols as a stimulants were added, as expected the addition of methanol has increased the yield (Tables3, 4 and 5). The explanation for how the methanol is acting as a stimulants is, addition of low molecular weight alcohols to the medium increases fungal tolerance to trace metals during fermentation.[30,31] In addition, methanol markedly depressed the synthesis of cell proteins in the early stage of cultivation[32] and also increased the metabolic activity of enzyme citrate synthase. When methanol concentration was further increased, it resulted in the decreased citric acid production (Tables 3, 4 and 5) because of the disturbance in fungal metabolism. Methanol has also some role in conditioning the mycelia without impairing their metabolism. Similar, type of work has also been carried out.[33] Zulay et al. (1995),[34] proved the use of methanol as a stimulant and butanol had adverse affect on the rate of citric acid fermentation.

Thus, yield of citric acid can be enhanced more by considering all other physical and chemical parameters. By doing so we can produce one of the important bulk producing organic acid, i.e., citric acid economically using a municipal waste, Pomegranate waste.

CONCLUSION

Citric acid which is one of the important organic bulk product is produced by Aspergillus niger MTCC 281 using Pomegranate and its waste separately. And the rate of production was compared with the rich carbohydrate source, i.e., sucrose as a substrate for the citric acid production. In order to increase the rate of production alcohols addition at the time of inoculation was done to check whether the three alcohols used were acting as a stimulant or the inhibitor for the rate of citric acid produced. The three alcohols used are methanol, ethanol and butanol, at different concentrations ranging from 0.5 to 2.5% v/v. Finally, it is proved that even with the municipal waste, i.e., Pomegranate waste we can produce one of the important organic acid, i.e., citric acid. Of the three alcohols used methanol at 1% v/v is proved to act as a stimulant and the ethanol and butanol are showing adverse effect. In the present work we have considered only the stimulant but other than this we have many parameters for the production of citric acid so, considering the other parameters we can increase the rate of yield even with Pomegranate waste.

REFERENCES

1. Vanags, J. & Viesturs, U. Local mixing intensity measurements for the food industry and biotechnology. Food Technol. & Biotechnol, 2001; 39(1): 59-65.
2. Ma, J.F. Role of organic acid detoxification of aluminum in higher plants. Plant & Cell, 2000; 41(2): 383-390.
3. Johnson, S. Do Mitochondria regulate cellular iron homeostasis through citric acid and haem production? Implication for cancer and other diseases. Med. Hypoth., 2003; 60(1): 106-111.
4. Karaklins, R., Lemba, J. & Ciesturs, U. Biotechnology for organic acid production. J. Biotechnol, 2001; 5(6): 281-283.
5. Fernando, A.V., Carlos, G.A. & Torres, N.V. Metabolism of citric acid production by Aspergillus niger. Biotechnol. & Bioengin, 2000; 70(1): 82-108.
6. Adham, N.Z. Attempts at improving citric acid fermentation by Aspergillus niger in beet molasses medium. Bioresource Technol, 2002; 84(1): 97-100.

7. Adachi, D.M., Toyama, H., Yamada, M., Shinagawa, E. & Matsushita, K. New developments in oxidative fermentation. Appl. Microbiol. & Biotechnol, 2003; 60: 643-653.
8. Ikram-UI, H., Ali, S., Qadeet, M.A. & Iqbal, J. Citric acid production by mutants of Aspergillus niger from cane molasses. Bioresource Technol., 2004; 93(2): 125-130.
9. Boling, S.D., Douglas, M.W., Snow, J.L., Parsons, C.M. & Baker, D.H. Citric acid does not improve phosphorus utilization in laying hens fed a corn-soybean meal diet. Poultry Sci., 2000; 79(9): 1335-1337.
10. Karaffa, L. & Kubicek, C.P. Aspergillus Niger citric acid accumulation: Do we understand this well working black box? Appl. Microbiol. & Biotecnol, 2003; 61; 189-196.
11. Hang, Y.D., Luh, D.S. & Woodams, E.E. Microbial production of citric acid by solid-state fermentation of kiwifruit peel. J.Food Sci., 1987; 52: 226-227.
12. Hang, Y.D. & Woodams, E.E. Grape pomace, a novel substrate to microbial production of citric acid. Biotechnol. Lett, 1985; 7: 763.
13. Roukas, T. Citric acid gluconic acid production from fig by Aspergillus niger using solid-state fermentation. J. Ind. Microbiol. & Biotechnol, 2000; 25(6): 298-304.
14. Wang,J.L. & Liu, P. Comparison of citric acid production by Aspergillus niger immobilized in gels and crysogels of polyacrylamide. J. Ind. Microbiol., 1996; 16: 351-353.
15. Maddox, I.S., Lu, M.Y. & Brooks, J.D. Citric acid production by Aspergillus niger in solid substrate fermentation. Bioresource Technol., 1995; 54: 235-239.
16. Yuguo, Z., Zhao, W. & Xiaolong, C. Citric acid production from the mash of dried sweet potato with its drugs by Aspergillus niger in an external-loop airlift bioreactor. Process Biochem, 1999; 35(3-4): 237-242.
17. Takita, F., Koyama, H. & Hara, H. Organic acid metabolism in aluminium phosphate utilizing cells. Cell Physiol. 40(5), (1999) 489-495.
18. Honecker, S., Bisping, B., Yang. Z. & Rehm, H.J. Influence of sucrose concentration and phosphate limitation on citric acid production by immobilized cells of Aspergillus niger. Appl. Microbiol. & Biotechnol, 1989; 31: 17-24.
19. Iwaki, Y., Kohashi, K., Eya, H., Honda, K. & Arai, Y. Measurements and correlation of oxygen solubilities in aqueous solutions containing salts and sugars. Kagaku Kogaku Ronbunsha, 1990; 16: 1247-1251.

20. Navaratnam, P., Arasaratnm, V. & Balasubramaniam, K. Channelling of glucose by methanol from citric acid production from Aspergillus niger. Worl J. Microbiol. & Biotechnol, 1998; 14(4): 559-563.
21. Ali, S., Ashraf, H. & Haq, I. Enhancement in citrate production by alcoholic limitation. Online. J. Boil. Scis, 2002; 2(2): 70-72.
22. Kubicek, C.P. The role of the citric acid cycle in fungal organic acid fermentations. Biochemical Sym, 1988; 54: 113-126.
23. Morse, Anthrone in Estimating Low Concentrations of Sucrose. Anal. Chem. 1947: 19(12): 1012–1013. DOI: 10.1021/ac60012a021.
24. Morris, D. L. Quantitative determination of carbohydrate with Dreywood's anthrone reagent. Science, 1948: 107: 254.
25. Ludwig and Goldberg, The anthrone method for the determination of carbohydrates in foods and in oral rinsing, J. Den. Res., 1956: 35(1): 90-4.
26. R.D. Williams, L. Wicks, H.R.Bierman, and W.H.Olmsted., Carbohydrate Values of Fruits and Vegetables., J.Nutr., 1940; 19: 593-604
27. Roukas, T. Carob pod, a new substrate for citric acid production by Aspergillus niger. App. Biochem. & Biotechnol, 1998; 74(1): 43-54.
28. Roukas, T. Citric acid production from carob pod by solid-state fermentation. Enzym. Microb. Technol, 1999; 24: 54-59.
29. Kristiansen, B., Mattey, M. & Linden, J. Citric acid Biotechnology. Taylor and Francis Inc. Philadelphia, 1999; 55-62.
30. Zakowska, Z. & Joloka, A. Toxic components of molasses. Acta Biotechnol, 1984; 4: 171-178.
31. Sanjay, k. and Sharma, P. A highly performance fermentation process for production of citric acid from sugarcane molasses. Journal of microbiology, 1994; 23: 211-217.
32. Moyer, A.J. Effect of alcohols on the mycological production of citric acid in surface and submerged culture. Appl. Microbial, 1953; 1: 1-7.
33. Navaratnam, P., Arasaratnm, V. & Balasubramaniam, K. Channelling of glucose by methanol from citric acid production from Aspergillus Niger. Worl J. Microbiol. & Biotechnol, 1998; 14(4): 559-563.
34. Zulay, M.P., Gisela, P. & Ferrer, J.R. Effect of methanol and ethanol on citric acid production by Submerged fermentation. Rev. Technol, 1995; 18(3): 321-332.