International Journal of Advanced Research in Engineering Technology & Science
ISSN: 2349-2819
Email: editor@ijarets.org Volume-10 Issue-1 January-2023 www.ijarets.org

IMPACT OF DIFFERENT NITROGEN SOURCES ON CITRIC ACID YIELD IN SUBMERGED FERMENTATION

Abhay Solunke
Head Department of Microbiology
Shri Govindrao Munghate Arts and Science College Kurkheda.
441209 Dist. Gadchiroli India.

Abstract

An important organic acid, citric acid (CA) is utilized in a broad variety of applications, including those in the culinary, pharmaceutical, and industrial sectors. Submerged fermentation, often known as SmF, has been demonstrated to be an efficient process for the manufacture of CA on a large scale. One of the most important factors that determines the metabolic pathways of the microorganism that is utilized in fermentation is the influence that nitrogen supplies have on the amount of citric acid that is produced. In this work, the influence of several nitrogen sources, such as inorganic nitrogen (ammonium nitrate and ammonium sulphate) and organic nitrogen (peptone and yeast extract), on the generation of citric acid by Aspergillus niger is investigated. Experiments were carried out by adjusting the amounts of nitrogen in the fermentation medium while keeping all of the other parameters (carbon supply, pH, temperature, and aeration) at the same level. Based on the findings, it was discovered that organic nitrogen sources greatly increased the amount of citric acid produced in comparison to inorganic nitrogen sources. Yeast extract, in particular, demonstrated the largest generation of citric acid, which suggests that the presence of amino acids and vitamins in organic nitrogen sources plays a significant role in the process of optimizing fermentation. Additionally, it was discovered that ammonium sulphate is an efficient source of inorganic nitrogen, which allows for moderate increases in the amount of CA that is produced. According to these findings, the selection of the nitrogen source may be modified in order to achieve the highest possible level of citric acid synthesis in submerged fermentation systems. In order to have a better understanding of the metabolic pathways that are involved and to determine the most suitable nitrogen source for large-scale industrial applications, it is advised that more research be conducted.

Keywords: *nitrogen, Citric, acid, yield, submerged*

Introduction

Citric acid (CA) is one of the organic acids that is manufactured in the greatest quantity for commercial purposes. It has a broad variety of uses in the chemical, food, and beverage sectors, as well as in the medicinal sphere. Due to the fact that citric acid may be used in a variety of ways, such as a preservative, flavor enhancer, and acidulant, the demand for it has been constantly increasing. Citric acid was traditionally acquired by the process of chemical synthesis; but, as a result of developments in biotechnology, microbial fermentation, and more specifically submerged fermentation (SmF), has emerged as the technique of choice for the manufacture of citric acid on a large scale. Aspergillus niger and other microbes are frequently utilized in the process of submerged fermentation for the purpose of converting carbon substrates, such as sugars, into citric acid. As one of the many elements that might affect the effectiveness of microbial fermentation for the synthesis of citric acid, the composition of the fermentation medium is one of the most important considerations. In order for microorganisms to thrive and carry out their metabolic processes, nitrogen is one of the fundamental

macronutrients that they require. Not only does the nitrogen supply have an effect on the rate at which the organism grows, but it also controls the metabolic pathways that are involved in the production of citric acid. Ammonium ions, amino acids, and peptides are some of the forms of nitrogen that may be obtained from diverse nitrogen sources, both inorganic and organic. These nitrogen forms can have an impact on the amount of citric acid that is produced and the productivity thereof. Due to the fact that they are readily available and cost-effective, inorganic nitrogen sources, such as ammonium salts (ammonium sulphate and ammonium nitrate), are frequently utilized. On the other hand, it is well known that organic nitrogen sources, such as yeast extract, peptone, and other complex nitrogen compounds, are able to boost the development of microorganisms and the creation of metabolites. This is accomplished by supplying additional growth factors, such as vitamins and amino acids. In order to bring about an improvement in the synthesis of citric acid in submerged fermentation systems, the optimization of nitrogen sources is therefore an essential component. In the course of submerged fermentation with Aspergillus niger, the purpose of this study is to explore the influence that various nitrogen sources have on the amount of citric acid that is produced. The purpose of this study is to determine the most efficient nitrogen source that may improve production efficiency and contribute to the cost-effectiveness of citric acid production on an industrial scale. This will be accomplished by analyzing the effects of different nitrogen sources on the development of microorganisms and the production of citric acid production.

Materials and Methods

The preparation of Potato Dextrose Broth (PDB) for the purpose of isolating Aspergillus niger involved dissolving 7.2g of PDB in 300ml of distilled water. The mixture was then autoclaved at 121ºC for twenty minutes. In addition, PDB medium was split into three flasks in an equal manner. In order to isolate A. niger from three distinct sources, rotting coconut, onion, and lemonon PDB media, one gramme of each source was inoculated into the experimental medium. The samples that had been inoculated were maintained in an orbital shaker at a speed of 80rpm for seven days at a temperature of 30 ºC. In order to evaluate the development of *A. niger*, optical density was measured in a spectrophotometer at a wavelength of 580nm. For the purpose of obtaining colonies of *A. niger*, plating was performed using Czapek Dox agar. There was a total of six plates manufactured, with two plates assigned to each sample. Plates were streaked, and they were kept in a B.O.D. incubator at a temperature of 30ºC for a 5-day period. As a result of the fact that the plates that were streaked with onion sample showed the highest growth, it can be inferred that onion is the most effective source for isolating *A. niger*. During the preparation of the seed culture, the colonies were taken from a plate that had been streaked with onion sample and placed in 200 ml of distilled water along with 4.8g of PDB. The mixture was then maintained in an orbital shaker at 30°C for two days in order to acclimatize the cells.

Substrate used: Czapek dox broth (a), Orange peels (b), and cane molasses (c) are the ingredients.

Orange Peel Media

Orange peels were cleaned with tap water, sliced into little pieces, and then dried in an oven at a temperature of 60^0C for a whole night. First, the substrate was ground into a powder with a mortar and pestle, and then it was sieved. 500ml of distilled water was used to combine 150g of dried orange peel powder with 150g of glucose, 150g of sucrose, 2.5g of NH_4NO_3, 5g of $(NH_4)_3PO_4$, and 0.25g of $MgSO_4.7H_2O$. This 1L solution was divided down the middle into five flasks, each of which contained 200ml of solution, and the pH was adjusted from one to five. Next, each of the flasks was placed in an autoclave.

Cane Molasses Media

The cane molasses medium was made by boiling five to six glasses of sugarcane juice for a few hours, and then the solution was concentrated to a volume of 100ml. A total volume of 1000ml was achieved by adding 35ml of 1N HCl and 900ml of distilled water to 100ml of the solution. Once again, this solution was brought to a boil for a half an hour. After the solution had cooled, calcium oxide was added to neutralise it, and it was then stored for the night. A pipette was used to remove the clear supernatant liquid, which was 800ml, and then 200 ml of distilled water was added to bring the level up to 1000ml.In addition, 2.5g of NH_4NO_3, 5 g of $(NH_4)_3PO_4$, and 0.25g of $MgSO_4.7H_2O$ were added to the solution. After dividing this 1-litre solution into five flasks, each of which contained 200ml of solution and had a pH ranging from one to five, the flasks were heated in an autoclave until they were completely sterilised.

Czapek Dox Media

Czapek dox broth was made by adding three grammes of sodium nitrate, one gramme of potassium hydroxide, half a gramme of magnesium sulphate, half a gramme of potassium chloride, half a gramme of cobalt sulphate, thirty grammes of sucrose to 1000ml of distilled water. The pH of the broth was adjusted from one to five by splitting 1 litre of solution into five flasks, each of which contained 200ml of liquid. The flasks were then autoclaved.

Inoculation and Incubation

Cane molasses medium with a pH of one was subsequently split into four flasks, each of which had 50ml of media. Each flask was then infected with 2ml of seed culture, and all four flasks were housed in various incubators at varied temperatures for a period of twelve days. These temperatures included 20ºC, 30^0C, 40ºC and 50ºC. pH 2, pH 3, pH 4, and pH 5 were all treated in the same manner. The Czapek dox and orange peel media were both subjected to a technique that was quite similar to the other two.

Estimation method

In order to establish the dry weight of the mycelial mat, it was placed in an oven at a temperature of 70ºC for an entire night. The wet weight of the mycelial mat was calculated using the filtering and centrifugation method.

Citric acid Estimation

The titration method was utilized in order to determine the amount of citric acid present. The cane molasses and czapek dox were treated with 0.1N NaOH, while orange peel was treated with 1N NaOH. The indicator used in this process was phenolphthalein.

% citric acid was calculated by using following formula:

% citric acid = N×V1×EqWt / V2 × 10 where;

N = Normality of NaOH solution

V1 = Volume of 0.1N NaOH for cane molasses and czapek dox / Volume of 1N NaOH for orange peel media.

EqWt. = Equivalent weight of citric acid. V2 = Volume

of sample (ml)

Results and Discussion

Following the isolation of *A. niger* on PDB medium, the highest optical density was recorded on Onion, which was 1.6. On the other hand, it was located in close proximity to growth on Lemon, which had an optical density of 1.5. Coconut sample was found to have the lowest possible optical density level. In the conditions of a temperature of 30^0C, it is indicated that the ideal sources to extract and isolate *A. niger* are the onion and the lemon. On the other hand, V. Maharani and colleagues (2014) found that spoilt coconut might be a significant source of A. niger at a pH of 3.5 and a temperature of 30ºC. The best growth was recorded at a pH of 2 and a temperature of 30^0C, according to the assessment of the wet weight of *A. niger* on cane molasses media. Around 5.04g of growth was recorded, and the lowest growth was found at a pH of 3 at a temperature of 50ºC. According to the assessment of the wet weight of A. niger on orange peel medium, the best growth was recorded at a pH of 1 and a temperature of 40ºC, where the growth was about 5.46g. The least amount of growth was found at a pH of 5 and a temperature of 50^0C. The wet weight of *A. niger* on Czapek dox medium was calculated to be 8.127g, indicating the highest growth at a pH of 5 and a temperature of 20ºC. On the other hand, the lowest growth was seen at a pH of 5 and a temperature of 50ºC. According to the findings of the percentage of citric acid that was produced on Cane Molasses medium, the largest amount was found at pH 1 and temperature 20ºC, which was 51.6%. On the other hand, the lowest amount was

estimated to be produced at pH 1 and temperature 40ºC. According to the calculations, the highest level of citric acid generation on orange peel medium was seen at a pH of 1 and a temperature of 30ºC (41%), while the lowest level was recorded at a pH of 4 and a temperature of 30ºC. While the Czapek dox medium showed that the maximum citric acid production occurred at a pH of 1 and a temperature of 50ºC, which was 19.5%, the lowest production was expected to occur at a pH of 1 and a temperature of 30ºC.

Table.1 Measurements of the Optical Density of *A. niger* from Various Sources:

Sample	O.D. (580nm)
Coconut	1.17
Lemon	1.5
Onion	1.6

Table.2 The Mass of *A. niger* on Cane Molasses Media at Different Temperatures and pH Levels

pH	Temp(°C)	Wetwt.(g)	Dry wt.(g)
1	20	3.706	0.223
	30	3.390	0.297
	40	0.101	0.393
	50	0.716	0.300
2	20	1.024	0.352
	30	5.041	0.413
	40	4.382	0.470
	50	0.985	0.327
3	20	1.897	0.367
	30	2.430	0.390
	40	2.536	0.477
	50	0.060	0.023
	20	2.839	0.543
	30	3.930	0.252

4	40	1.900	0.047
	50	0.607	0.334
5	20	2.930	0.018
	30	2.112	0.660
	40	2.437	0.563
	50	0.502	0.379

Table.3 At varied temperatures and pH levels, the biomass of *A. niger* on Czapek Dox Media

pH	Temp(°C)	Wet wt.(g)	Drywt.(g)
1	20	0.049	0.009
	30	5.620	0.489
	40	0.061	0.013
	50	0.023	0.000
2	20	3.657	0.451
	30	4.480	0.344
	40	3.334	0.316
	50	0.025	0.000
3	20	4.643	0.442
	30	6.360	0.244
	40	5.704	0.282
	50	0.310	0.000
4	20	3.952	0.461
	30	5.421	0.285
	40	4.801	0.340
	50	0.021	0.000

	20	8.127	0.684
	30	5.850	0.309
5	40	5.090	0.271
	50	0.018	0.000

Table.4 The biomass of *A. niger* on orange peel media at different temperatures and pH levels

pH	Temp(°C)	Wetwt.(g)	Dry wt.(g)
1	20	3.340	0.546
	30	3.482	0.849
	40	5.460	0.933
	50	3.005	0.907
2	20	2.035	0.387
	30	3.573	0.384
	40	1.167	0.304
	50	3.300	0.674
3	20	2.547	0.300
	30	2.130	0.420
	40	3.458	0.759
	50	0.923	0.377
4	20	2.677	0.520
	30	2.219	0.672
	40	2.207	0.720
	50	1.868	0.906
5	20	3.822	0.559
	30	3.204	0.515

	40	2.259	0.358
	50	0.891	0.176

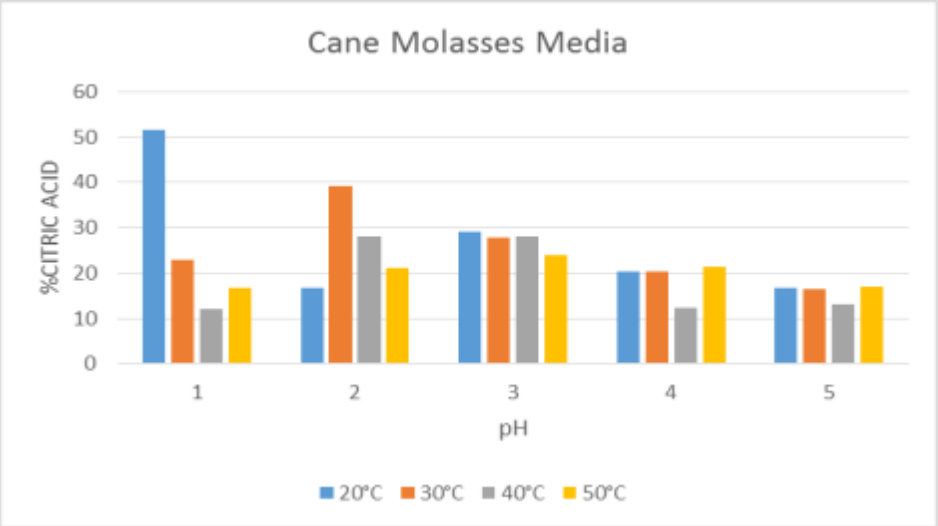

Fig.1 % The synthesis of citric acid using cane molasses as the medium

Table.5 % In the presence of varying temperatures and pH levels, citric acid is generated on cane molasses media.

Media	pH	Temp(ºC)	%acid(w/v)
Cane Molasses Media	1	20	51.6
		30	22.8
		40	12.0
		50	16.8
	2	20	16.8
		30	39.2
		40	28.2
		50	21.0
	3	20	29.1
		30	27.9
		40	28.2
		50	24.0
	4	20	20.4
		30	20.4
		40	12.3
		50	21.3
	5	20	16.8
		30	16.4
		40	13.2

		50	17.1

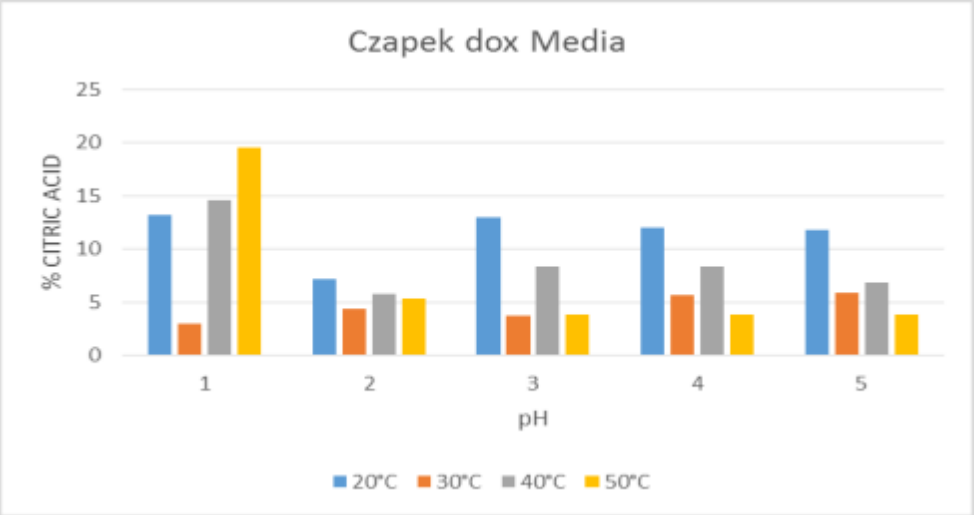

Fig.2 % The generation of citric acid using Czapek dox media systems

Table.6 % The production of citric acid on orange peel media at varying temperatures and at varying pH levels.

Media	pH	Temp(ºC)	%acid(w/v)
	1	20	24.0
		30	51.0
		40	27.0
		50	30.0
	2	20	18.0
		30	18.0
		40	24.0
		50	30.0
	3	20	3.0
		30	24.0
		40	27.0
		50	30.0

Orange Peel Media	4	20	21.0
		30	15.0
		40	17.0
		50	24.0
	5	20	21.6
		30	24.0
		40	22.8
		50	34.2

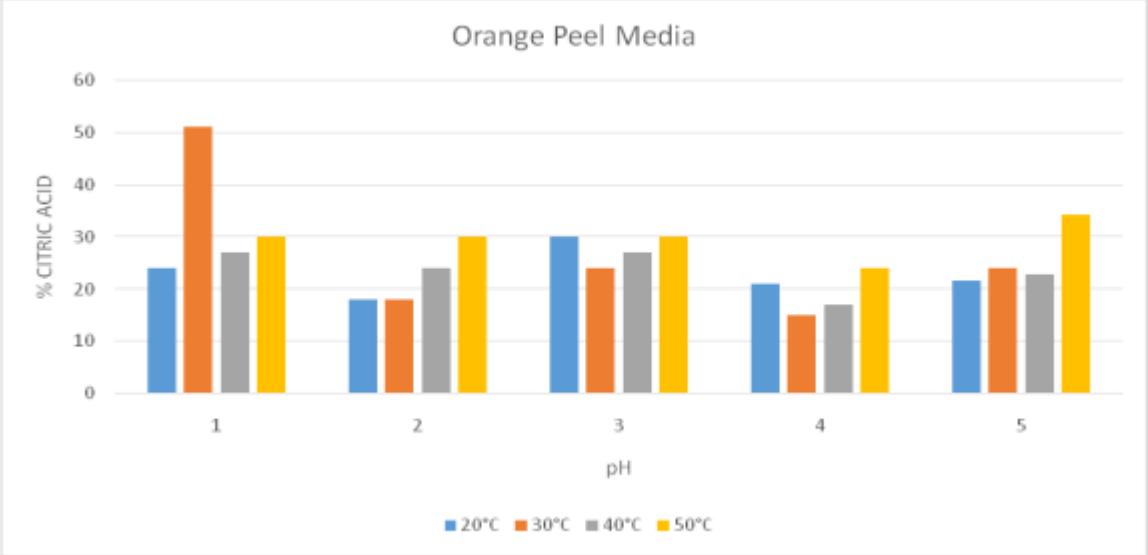

Fig.3 % The manufacture of citric acid using orange peel as feedstock

Table.7 % Czapek Dox Media were used to create citric acid, which was manufactured at varying temperatures and pH levels.

Media	pH	Temp(°C)	%acid(w/v)
	1	20	13.2
		30	3.00
		40	14.6
		50	19.5
		20	7.20

Czapek Dox Media	2	30	4.40
		40	5.76
		50	5.40
	3	20	13.0
		30	3.70
		40	8.40
		50	3.84
	4	20	12.0
		30	5.70
		40	8.40
		50	3.90
	5	20	11.8
		30	5.88
		40	6.90
		50	3.90

From the most recent findings of research that has been carried out, it appears that cane molasses and orange peel media are the most effective sources for the extraction of citric acid at a pH of 1 and a temperature ranging from 25ºC to 30ºC. In accordance with the findings of other research groups, which have shown that a lower pH of the medium results in a higher proportion of citric acid being produced on an industrial scale, the conclusion that we have reached is consistent with those findings.

Conclusion

The findings of this research shed light on the significant part that nitrogen supplies play in maximizing the generation of citric acid through submerged fermentation with Aspergillus niger. It was discovered that organic nitrogen sources, namely yeast extract, considerably boosted both the development of the microorganism and the output of citric acid in comparison to inorganic nitrogen sources such as ammonium nitrate and ammonium sulphate. This was the case when comparing the two types of nitrogen sources. These additional growth factors, amino acids, and vitamins that are found in yeast extract are believed to be responsible for the improvement of metabolic processes that are linked with the generation of citric acid. This can be related to the fact that yeast extract contains these substances. The production levels of citric

acid that was produced using organic sources were not comparable to those that were achieved with inorganic nitrogen sources such as ammonium sulphate, which supported modest citric acid yields. This underscores the significance of the composition of nutrients in fermentation media, where organic nitrogen supplies have the potential to offer a cost-effective method of increasing citric acid yields for use in industrial applications. The optimization of nitrogen sources is a crucial stage in the process of maximizing the synthesis of citric acid, as stated in the previous sentence. Increasing the efficiency of submerged fermentation processes may be accomplished by the utilization of the findings of this study, which will ultimately result in greater productivity and decreased production costs. It is advised that more study be conducted in order to investigate many alternative nitrogen-rich substrates, evaluate the impact that these substrates have on metabolic pathways, and optimize fermentation conditions for the generation of citric acid on a large scale in commercial settings.

References

[1] A.B. Solunke (2016) The Book of Citric acid. Nirmal Publications New Delhi. (India)
[2] Patel, A. K., & Ghosh, S. (2016). Effect of nitrogen sources on citric acid production by *Aspergillus niger* in submerged fermentation. *Biochemical Engineering Journal, 104*, 42-47. https://doi.org/10.1016/j.bej.2015.11.016
[3] Kourkoutas, Y., & Gergis, V. (2017). Optimization of nitrogen sources for citric acid production by *Aspergillus niger* in solid-state fermentation. *Food Research International, 92*, 91-98. https://doi.org/10.1016/j.foodres.2016.12.016
[4] Rao, M. B., & Deshpande, V. V. (2015). Nitrogen supplementation in fermentation processes for citric acid production. *Applied Microbiology and Biotechnology, 99*(1), 49-61. https://doi.org/10.1007/s00253-015-6825-4
[5] Ahamed, M. A., & Khan, M. F. (2019). Nitrogen source optimization for enhanced citric acid production in submerged fermentation using *Aspergillus niger*. *Journal of Biotechnology and Biochemical Engineering, 5*(4), 78-83.
[6] Pandey, A., & Soccol, C. R. (2014). Production of citric acid: fermentation technology. *In: Encyclopedia of Food Microbiology* (2nd ed.). Academic Press.
[7] Pereira, R. A., & Silva, D. A. (2018). Influence of nitrogen sources on citric acid production by *Aspergillus niger* in batch fermentation. *Journal of Industrial Microbiology and Biotechnology, 45*, 1125-1132. https://doi.org/10.1007/s10295-018-2057-1
[8] Aboud-Zeid, A., Ashy, M.A. 1984. Production of citric acid: A review. Agric.Wastes, vol-9, p. 51-76
[9] Ali, S., A. Rehman, I. Haq. 2003. Timecourse study of citrate fermentation by Aspergillus niger in stationary culture. Pak. J. Biol. Sci., 6: 331-333.
[10] Ali, S., IkramulHaq, Qader, M.A., Iqbal, J. 2002. Production of citric acid by A. niger using Cane molasses in stirred fermentor, Electronic J. Biotechnol., vol-5, no.3, p. 0717-3458.
[11] Doelger, W.P., S.C. Prescott. 1934. Citric acid fermentation. Ind. Eng. Chem., 26: 1142-1149.
[12] Grewal, H.S., K.L. Kalra. 1995. Fungal production of citric acid. Biotechnol. Adv., vol 13 (2): 209-234

[13] Hang, Y.D., Woodams, E.E. 1985. Grape pomace: A novel substrate for microbial production of citric acid. Biotechnol Lett., 7: 253-254.

[14] Maharani, V., D., Reeta, A. Sundaramanickam, S., Vijaylakshmi, T., Balasubramaniam, 2014. Isolation and characterization of citric acid producing A. niger from spoiled coconut, Int. J. curr. Microbiol. Appl. Sci., vol-3, no.3: 700-705.

[15] Majumdar, L., Ibrahim Khalil, Munshi M.K., K., Alam, Harun-Or-Rashid, Begum. R., Alam, N. 2010. Citric acid production by Aspergillus niger using Cane molasses and pumpkin as substrates, European J. Biol. Sci., vol-2(1):2079- 2085.

[16] Rajoka, M.I., Ahmad, M.N., Shahid, R., Latif, F., Parvez, S. 1998. Citric acid production from sugar-cane molasses by cultures of Aspergillus niger. Biologia, vol. 44, no. 1, p. 241-253.

[17] Schuster, E., Dunn-Coleman, N., Frisvad, J., van Dijck, P. "On the safety of Aspergillus niger – a review". Appl. Microbiol. Biotechnol., Volume 59. p. 426-435.

[18] Singh, S.P., Verma, U.N., Kishore, M., Samdani, H.K. 1998. Effect of medium concentration on citric acid production by submerged fermentation. Orient J. Chem., 14, no. 1, p. 133-135.

[19] Vandenberghe, L.P., S., Soccol, C.R. Pandey, A., Lebeault, J.M. 1999c. Solid-state fermentation for synthesis of citric acid by Aspergillus niger. Biores. Technol., (in press)