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Production of Citric Acid in Basal Coffee Husk Medium by *Aspergillus niger* under Solid State Fermentation

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Abstract: Citric acid is the most important organic acid produced in tonnage by fermentation. In the present study *Aspergillus niger* is used for the production of citric acid in basal coffee husk medium under solid state fermentation. Addition of methanol (3% v/w) to coffee husk medium in optimum moisture 45%, *p*H 4.5 and supplemented with trace elements had shown significant improvement in citric acid production. 187.54g citric acid/kg dry coffee husk was accumulated at 72 h resulting in 78.14% and 83.36% conversions based on the amount sugar of initially present in coffee husk and sugar consumed during fermentation respectively, with 97.73% sugar utilization.

Key words: Citric Acid · Coffee Husk Medium · Solid State Fermentation · *Aspergillus niger*

INTRODUCTION

Recently a wide range of citric acid production has grape pomace, apple pomace, wheat bran, coffee been reported in response to different levels of nutrient supplementation [1, 2]. Citric acid (2-hydroxy-propane-1, 2,3-tricarboxylic acid) originally described as a constituent from citrus fruits and known as an intermediate of citric acid cycle (TCA) combines a pleasant taste with low toxicity and palatability and thus became an ubiquitous food additive [3]. It has multitude of usage in food and beverages, pharmaceuticals, chemical, cosmetic and other miscellaneous industries [3- 6]. The discovery of citric acid has been credited to the 8th century Arab-Yemeni (Iranian born) alchemist Jabir Ibn IIayyan [7].

One of the most significant parameters explaining the commercial success of citric acid is the huge market size. The production of citric acid estimated at 1.4 million tons in 2004, a number that far exceeds the production of any other organic acid made by fermentation by BCC Report, 2005. Only mutants of *A. niger* are used for commercial production of citric acid. The reason being that compared to Penicillium strains, the Aspergilli produce more citric acid per unit time, moreover, the production of undesirable side products such as oxalic acid, isocitric acid and gluconic acid, can be more easily and efficiently suppressed in these mutants of *A. niger* [8]. Citric acid can be produced by three different fermentation routes: 1) Submerged Fermentation (SmF); 2) Liquid Surface Fermentation (LSF) and 3) Solid State Fermentation (SSF).

Coffee (Coffea Sp) is one of the most important agricultural commodities in the world. *C. arabica* and *C. robusta* are the two principal varieties of the genus cultivated all over the world for commercial production [8]. The chief by-products of coffee processing coffee husk, pulp, leaves and spent ground are generated in more than two million tons per annum [9]. These by-products are simply disposed as waste without proper treatment thereby leading to environmental pollution. Coffee husk, a solid residue generated in the processing of coffee by the dry method, contains about 25% fermentable sugars on dry weight basis [10]. This valuable by-product has been reported to be an efficient carbon source for

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the production of citric acid by *A. niger* under SSF [8]. Very few reports are available on production of citric acid using coffee husk as substrate under SSF.

The present study on maximum production of citric acid in basal coffee husk medium by *A. niger* under solid state fermentation and optimization of several parameters.

MATERIALS AND METHODS

Substrate: Coffee husk was procured from a local coffee processing unit, substrate were obtained in a quantity sufficient for all the experiments in a single batch. The sugar content of substrate was analyzed by phenol-sulphuric acid method of Dubois *et al.* [11] and Glucose in coffee husk waste was determined by the DNS method of Miller [12].

Pretreatment of Coffee Husk: Crude Coffee husk were thoroughly washed with deionized water to remove loosely sticking flour or dirt, filtered, dried at 80°C. The dried substrates were pulverized in a dry grinder and passed through standard sieves with a pore size of 0.5mm. This pretreatment was done to increase surface area of cellulosic and lignocellulosic residues, to reduce crystallinity and hence to improve substrate solubility for microbial attack and penetration [13-16].

Aspergillus Niger: Soil samples were collected from rotten fruit dump outside whole sale fruit stall, Shimoga, through serial dilution and plated on Czapekdox agar medium supplemented with 0.8% sodium deoxycholate and 0.04% bromocresol purple. The petridishes were incubated in an incubator at 30°C for 48h and well-separated colonies, resembling the colony characteristics of *Aspergillus niger* [17- 19] were picked up for subculture on Potato Dextrose Agar slants. The organic acid producing ability of the strains was tested by plating on agar medium incorporated with calcium carbonate and Czapekdox agar medium supplemented with 0.8% sodium deoxycholate and 0.04% bromocresol purple.

Inoculum Preparation: Spore suspension was prepared from a 168h old slant culture grown on PDA, by adding 10ml of sterile distilled water containing 0.01% Tween-80 and scraping the spores with a sterile spatula. 1ml of the spore suspension (about 1X 10^7 spores/ml) was used to inoculate each flask of SSF medium.

Solid State Fermentation (SSF): 10g of Coffee husk (CH) (particle size: 0.5mm) was charged into 250ml Erlenmeyer flasks and moistened with 0.2M HCl (initial moisture content: 50% and initial *p*H: 4.0) autoclaved at 121°C for 30 minutes.

Standardization of Medium Parameters: Few selected parameters were studied for their influence on citric acid production using CH medium. The parameters found beneficial were incorporated one by one in the medium during the standardization of next parameter. The values reported are the average values of triplicate experiments. CH medium initial moisture content maintained at 45%, *p*H 4.5, supplemented with standardized trace elements (with mixture of iron, copper and zinc at 1.0, 0.3 and 0.2 ppm, respectively) was enriched with ammonium nitrate (0.05-0.2% w/w). Sucrose was added to CH medium at different concentrations (5-20% w/w) to boost carbon substrate. Next methanol was added at 3% v/w level to the fermentation media containing 10g of coffee husk. To this add 1ml of the spore suspension (about 1X 10^7 spores/ml) and incubate at 30°C in an incubator under stationary conditions.

Analytical Techniques: The sugar content of substrate was analyzed by phenol-sulphuric acid method of Dubois [11]. The citric acid content of the fermented coffee husk was determined by the colorimetric method [20]. The pH of the fermented mash was measured using digital pH meter [13]. The data obtained in the above experiments were subjected to one-way ANOVA followed by Turkey's multiple comparison tests. Citric acid yield was expressed as g citric acid $100g^{-1}$ sugar consumed and sugar utilization was calculated by dividing sugar consumed during fermentation by initial sugar and multiplying the result by 100.

RESULTS

The kinetic parameters of *A. niger* RCNM 17 during SSF of citric acid in basal CH medium (initial moisture 50% and *p*H 4.0) is shown in Table-1. Highest titer of citric acid 82.38g kg^{-1} dry CH was produced at 72h with 61.19% sugar utilization, which works out to be 34.32% and 56.09% conversion based on the amount of sugar initially present in CH medium and sugar consumed during fermentation respectively. The lowest *p*H (2.3) was recorded with highest titer of citric acid and the biomass growth (in terms of glucosamine) was increased with increase in fermentation time (Fig.1).

Table 1:Kinetic parameters of *A. niger* RCNM 17 during SSF of citric acid in basal coffee husk (CH) medium

Fermentation Time (h)	Citric acid (g/kg dry CH)	Residual sugars (g/kg dry CH	*p*H
24	13.76	209.12	3.4
48	34.57	164.48	2.8
72	82.38	93.14	2.3
96	69.42	80.26	2.5
120	43.14	72.35	2.8

Table 2: Influence of initial *p*H on SSF of citric acid by *A. niger* RCNM 17 in CH medium

*p*H		Citric acid and residual sugars (g/kg dry CH)				
Fermentation time (h)-		24	48	72	96	120
2.0	Citric acid	0.8	4.68	17.39	41.36	24.35
	Res. sugars	221.23	202.85	173.28	141.32	112.23
3.0	Citric acid	3.8	18.65	51.33	32.63	20.02
	Res. sugars	215.32	182.29	141.56	132.23	120.68
4.0	Citric acid	12.65	36.87	83.02	62.35	41.23
	Res. sugars	201.25	162.34	93.34	83.36	72.68
4.5	Citric acid	24.36	56.47	115.41	102.32	78.35
	Res. sugars	186.32	119.34	60.34	51.22	43.67
5.0	Citric acid	19.03	40.58	92.34	64.22	49.38
	Res. sugars	190.68	123.56	78.69	63.32	51.38
6.0	Citric acid	11.47	27.64	54.68	41.25	16.35
	Res. sugars	213.25	168.44	69.38	48.67	42.13
7.0	Citric acid	3.25	11.68	28.33	41.68	30.54
	Res. sugars	218.58	160.58	120.66	69.28	49.66

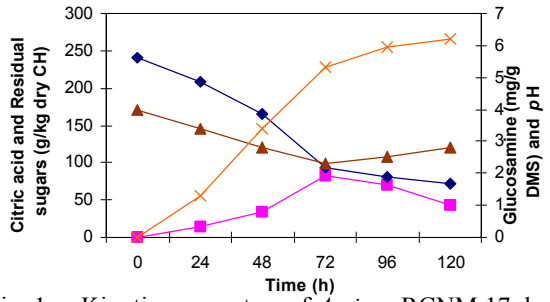

Fig. 1: Kinetic parameters of *A.niger* RCNM 17 during Solid State Fermentation (SSF) of citric acid in basal coffee husk (CH) medium. Citric acid (■), residual sugars (♦), glucosamine (×), *p*H (▲).

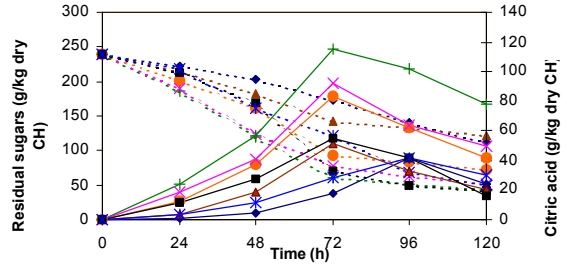

Fig. 4.2: Influence of initial *p*H on SSF of citric acid by *A.niger* RCNM 17 in CH medium. Citric acid (-) and Residual sugars (...). *p*H 2 (♦), *p*H 3 (_), *p*H 4 (•), *p*H 4.5 (+), *p*H 5 (×), *p*H 6 (■), *p*H 7 (*).

Influence of Initial pH: A. niger RCNM 17 produced maximum amount of citric acid (115.41g citric acid kg^{-1} CH) at optimum *p*H 4.5 utilizing 74.85% sugar (Table-2). The yields of citric acid were 48.08% and 64.23% based on the amount of sugar initially present in CH medium and sugar consumed during fermentation respectively. Citric acid production was lower at all other *p*H values (Fig. 2)

Influence of Initial Moisture Level: Highest titer of citric acid (128.87g citric acid kg^{-1} CH) was obtained with 45% initial moisture level (Table-3). The yields were 53.69% and 69.53% based on the amount of sugar initially present in CH and sugar consumed during fermentation respectively with 77.22% sugar utilization. Citric acid production was lower with all other moisture (Fig.3).

Effect of Metal Ions: Addition of Fe^{2+}, Cu^{2+} and Zn^{2+} (1mg, 0.3mg and 0.2mg kg^{-1} CH respectively) to the CH medium (initial moisture 45% and *p*H 4.5) resulted in further improvement in citric acid production (Table-4). Maximum citric acid production was obtained at 72h (146.34g citric acid kg^{-1} CH) (Fig.4). The yields of citric acid were 60.17% and 71.24% based on the amount of sugar initially present in CH and sugar consumed during fermentation respectively with 85.69% sugar utilization.

Table 3:Influence of initial moisture level on SSF of citric acid by *A. niger* RCNM 17 in CH medium

Moisture level (%w/w)		Citric acid and residual sugars (g/kg dry CH)				
Fermentation time (h)		24	48	72	96	120
30	Citric acid	4.09	18.32	29.65	51.66	42.28
	Res. sugars	223.68	187.98	156.36	109.58	73.98
40	Citric acid	17.35	36.28	82.67	63.34	47.28
	Res. sugars	203.57	166.35	93.57	81.57	70.38
45	Citric acid	28.67	57.05	128.87	103.24	79.68
	Res. sugars	200.36	119.87	54.66	43.26	39.87
50	Citric acid	23.58	55.32	115.98	98.03	70.35
	Res. sugars	184.72	115.30	65.34	49.36	41.32
60	Citric acid	15.79	38.65	71.06	43.28	29.06
	Res. sugars	201.68	179.87	67.96	53.25	50.32
70	Citric acid	6.38	19.67	41.02	30.68	20.88
	Res. sugars	199.28	165.20	94.32	59.87	43.28

Table 4: Influence of trace elements on SSF of citric acid by *A. niger* RCNM 17 in CH medium

	Medium without added trace elements			Medium with added trace elements		
Ferementation time (h)	Citric acid (g/kg dry CH)	Residual sugars (g/kg dry CH)	*p*H	Citric acid (g/kg dry CH)	Residual sugars (g/kg dry CH)	*p*H
24	25.46	180.28	3.6	31.54	184.35	3.5
48	53.89	116.57	3.0	69.87	110.58	2.8
72	128.09	51.98	2.2	146.34	34.33	2.0
96	99.78	43.57	2.4	123.34	21.21	2.3
120	70.58	37.29	2.7	86.57	19.56	2.5

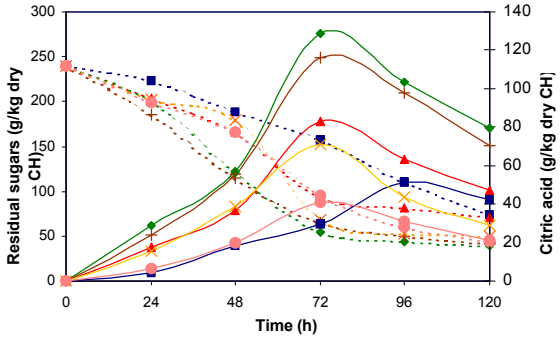

Fig. 3: Influence of initial substrate moisture level (%w/w) on SSF of citric acid by *A. niger* RCNM 17 in CH medium. Citric acid (-) and Residual sugars (...). Moisture level at 30% (■), 40% (_), 45% (®), 50% (+), 60% (×) and 70% (?).

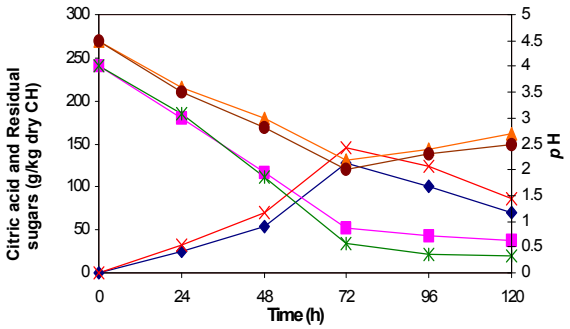

Fig. 4: Influence of trace elements on SSF of citric acid by *A.niger* RCNM 17 in CH medium. Citric acid (®), residual sugars (■) and *p*H (_) in medium without added trace elements and Citric acid (×), residual sugars (*) and *p*H (?) in medium with added trace elements.

Influence of Methanol: Addition of methanol (3% v/w) to CH medium (initial moisture 45%, *p*H 4.5 and supplemented with trace elements) had shown significant improvement in citric acid production (Table-5). Maximum amount of citric acid (187.54g kg^{-1} dry CH) was accumulated at 72 h resulting in 78.14% and 83.36% conversions based on the amount sugar of initially present in CH and sugar consumed during fermentation respectively, with 97.73% sugar utilization (Fig.5).

DISCUSSION

Coffee husk, a solid residue generated in the processing of coffee by the dry method, poses disposal problems and causes environmental pollution. It has been reported to contain about 25% fermentable sugars on dry weight basis [10]. This valuable by-product has been reported to be an efficient carbon source for the

Table 5: Influence of methanol (3%v/w) on SSF of citric acid by *A. niger* RCNM 17 in CH medium

	CH medium without methanol		CH medium with methanol (3%v/w)	
Fermentation time (h)	Citric acid (g/kg dry CH)	Residual sugars (g/kg dry CH)	Citric acid (g/kg dry CH)	Residual sugars (g/kg dry CH)
24	35.15	183.35	47.38	180.21
48	65.32	131.58	78.54	129.25
72	146.28	28.33	187.54	15.03
96	124.43	22.21	132.50	10.21
120	70.25	11.56	93.41	7.00

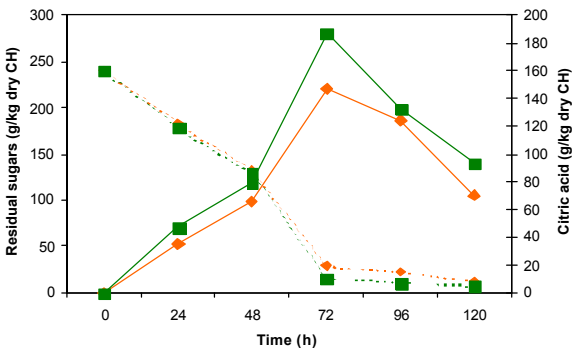

Fig. 5: Influence of methanol on SSF of citric acid by *A. niger* RCNM 17 in CH medium. Citric acid (-) and Residual sugars (...). Citric acid and residual sugars (♦) in medium without methanol. Citric acid and residual sugars (■) in medium with methanol (3%v/w).

production of citric acid by *A. niger* under SSF [8]. Very few reports are available on production of citric acid using coffee husk as substrate under SSF. In the present study, the data indicate that about 187 g citric acid is produced per kg dry coffee husk in the standardized medium, conversion reaching about 83% based on sugar consumed *A. niger* RCNM 17 could produce 82.38g citric acid/kg dry CH consuming 61.19% sugar. Shankaranand and Lonsane [10] reported *A. niger* CFTRI 30 produced 150g citric acid per kg dry coffee husk. Hence physicochemical factors affecting citric acid fermentation were optimized to improve the efficiency of *A. niger* RCNM 17 to give greater yields of citric acid. *A. niger* RCNM 17 produced highest titer of citric acid at optimal pH 4.5 (Table-2, Fig. 2) resulting in 64.23% conversion based on sugar consumed during fermentation. In SmF, low *p*H values (around 2.0) are generally employed. An extrinsic parameter, such as *p*H acts synergistically with other environmental parameters in addition to being a regulatory parameter in biotechnological process [22]. In literature, use of different initial substrate moisture levels has been reported for different substrates. For example; 65% for kiwi fruit peel (Hang *et al., s*1987), 73% for apple pomace [23], 65-75% for grape pomace [24], 75% for bagasse impregnated with concentrated liquor of pineapple waste [25] and 65% for carob pods [26]. In the present study maximum yield of citric acid was obtained at 45% initial moisture level (Table-3, Fig. 3). Citric acid production was increased to 146.34g/kg dry coffee husk when the medium was supplemented with metal ions viz., Fe^{2+}, Cu^{2+} and Zn^{2+} at 1mg, 0.3mg and 0.2mg/kg CH respectively (Table-4, Fig. 4). Similarly, consumption of sugar increased to 85.69% with simultaneous increases in conversions to 60.17% and 71.24% based on the total initial sugar and sugar consumed during fermentation respectively. Coffee husk used in the present study contained 4.2% ash. Coffee husk which contains 4.4 to 5.6% ash on a dry weight basis, slightly less than that in CWB, should be a more suitable substrate for the production of citric acid, at least in SSF system. Addition of methanol (3%v/w) increased citric acid production by 2.27% compared to basal CH medium (Table-5, Fig.5). The conversions were 78.14% and 83.36% based on amount of sugar present in the medium and sugar consumed during fermentation respectively. The influence of methanol on increasing citric yields appears to be a general phenomenon with the strains of *A. niger* and the use of methanol has become a common practice. Methanol is not assimilated by *A. niger* and its exact role in stimulating the production of citric acid is still not known. It is likely that methanol affects permeability properties of the mould and enables greater excretion of citric acid [5]. Citric acid production was maximum with 10% v/w inoculum ($1x10^7$ spores/ml). Spore viability and germination properties are known to change with the age of inoculums [27]. Low inoculum density might result in decreased amount of biomass, whereas higher inoculum density levels cause fierce competition for nutreients [14].

CONCLUSION

One of the greatest challenges in biotechnology is the development of suitable technology to enable cellulosic wastes (domestic, industrial and agricultural)

to be used as substrates for fermentation processes. Coffee husk (CH), a solid residue generated in the processing of coffee by the dry method contains 24% fermentable sugars on dry weight basis. *A. niger* RCNM 17 produced 82.38g of citric acid kg^{-1} dry CH at 72 h in basal CH medium. The present study indicated that agro industrial wastes used have a great potential as solid substrates for citric acid production by *A. niger* RCNM 17 in SSF system and also established the superiority of SSF system over traditionally used SmF and LSF systems. *A. niger* RCNM 17 could perform very well under Solid Substrate Fermentation conditions and supported maximum production of citric acid under optimal conditions of fermentation.

REFERENCES

1. Imandi, S.B., V.V.R. Bandaru, S.R. Somalanka and H.R. Garapati, 2007. Optimization of medium composition for the production of citric acid from byproduct glycerol using Doehlert experimental design. Enzyme. Microbial. Techno, l(40): 1367-1372.
2. Bari, M.N., M.Z. Alam, S.A. Muyibi, P. Jamal and A. Mamun, 2009. Improvement of production of citric acid from oil palm empty fruit bunches: Optimization of media by statistical experimental Designs Biores. Technol., 100: 3113-3120.
3. Rohr, M.A., 1998. Century of Citric acid Fermentation and Research. Food Technol. Biotechnol, 36: 163-171.
4. Meyrath, J., 1967. Citric acid production. Process biochemistry, pp: 25-27.
5. Kapoor, K.K., K. Chaudhary and P. Tauro, 1982. Citric acid, In "Prescott and Dunn's industrial microbiology". 4th edn. 709-711, G. Reed (Ed), AVI publishing Co, Westport, CT, USA.
6. Blair, G. and P. Staal, 1993. In Kirk-Othmer's Encyclopedia of chemical technology. John Wiley and Sons, Inc, New York, 6: 354.
7. http://en.wikipedia.org/wiki/citricacid.
8. Pandey, A., C.R. Soccol, A. Jose, Rodriguez-Leon and P. Nigam, 2001. In Solid State Fermentation in Biotechnology: Fundamentals and Applications, 1st Edn. Asia Tech Publishers INC New Delhi, pp: 120.
9. Pandey, A., C.R. Soccol, P. Nigam and V.T. Soccol, 2000a. Biotechnological potential of agroindustrial residues. I: Sugarcane bagasse. Bioresource Technol, 74: 69-80.
10. Shankaranand, V.S. and B.K. Lonsane, 1994. Coffee husk: an inexpensive substrate for production of citric acid by Aspergillus niger in a Solid State Fermentation system. World J. Microbiol. Biotechnol. 10(2): 165-168.
11. Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Roberts and F. Smith, 1956. Colorimetric method for the determination of sugar and related substances. Anal. Chem, 25: 350-354.
12. Mille, G.L., 1959. "Use of Dinitrosalicylic acid reagent for determination of reducing sugar". Anal.Chem, 31: 426-428.
13. Raimbault, M. and D. Alazard, 1980. Culture method to study fungal growth in Solid Fermentation. European J. Appl. Microbial Biotechnol, 9: 199-209.
14. Kaur and T. Satyanarayana, 2004. Production of extracellular pectinolytic, cellulolytic and xylanolytic enzymes by thermophilic mould Sporotrichum thermophile Apinis in Solid State Fermentation. Ind. J. Biotechnol, 3: 552-557.
15. Rasalkar, A.A., B.K. Salunke, R.Z. Sayyed, A.B. Chaudhari and S.B. Chincholkar, 2002. Solid State Cultivation of Curvularia lunata for transformation of Rifamycin B to S. Ind. J. Experiment. Biol., 40: 930-933.
16. Broder, J. and J.W. Dand Barrier, 1990. Producing fuels and chemicals from cellulose crops, in: Janick and Simon. J.E. (Eds,). Advances in new crops. Timber Press, Portland, pp: 257-259.
17. Alexopoulos, D.J. and E.S. Beneke, 1962. Laboratory Manual for Introductory Mycology. Surgess Publishing Company, Minneapolis 15, Minn, pp: 116.
18. Malloch, D., 1981. Moulds: Their Isolation, Cultivation and Identification. University of Toronto press. Toronto buffalo London, pp: 51-61.
19. www.doctorfungus.org/thefungi/aspergillus_spp.htm-97k
20. Marier, J.R. and M. Boulet, 1958. Direct determination of citric acid in milk with an improved pyridine acetic anhydride method. J. Dairy Sci., 4: 1683-1692.
21. Pandey, A., 2004. Alpha amylase from a fungal culture grown on oil cakes and its properties. Brazilian Archives of Biology and Technology, 47(2): 309-317.
22. Lonsane, B.K. and M.V. Ramesh, 1990. Production of bacterial thermo stable ∝-amylase by Solid State Fermentation: A potential tool for achieving economy in enzyme production and starch hydrolysis. Adv. Appl. Microbiol, 35: 1-56.
23. Hang, Y.D., B.S. Luh and E.E. Woodams, 1987. Microbial production of citric acid by Solid State Fermentation of kiwi fruit peel. Journal Of Food Science, 52: 226-227.
24. Hang, Y.D. and E.E. Woodams, 1984b. Apple pomace: A potential substrate for citric acid production by Aspergillus niger. Biotechnol Lett, pp: 763-764.

25. Usami, S. and N. Fukutomi, 1977. Hakkokogaku Kaishu, 55: 44.
26. Roukas, T., 1999. "Citric acid production from carob pod by Solid State Fermentation. Enz. Microb. Technol, 24(1-2): 54-59.
27. Sussman, A.S. and H.O. Halvorson, 1966. Spores: Their dormancy and germination. Academic press, New York.