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Solid state fermentation of BSG for citric acid production

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Brewers' spent grain (BSG) is the major by-product of the brewing industry, representing around 85 % of the total by-products generated. BSG is a lignocellulosic material containing about 17 % cellulose, 28 % non-cellulosic polysaccharides, chiefly arabinoxylans, and 28 % lignin. Nevertheless, due to its high content of protein and fibre (around 20 and 70 % dry basis, respectively), it can also serve as an attractive adjunct in human nutrition. The present investigation was carried out on citric acid production from Brewer's spent grain using *Aspergillus niger*. The chemical qualitative characterization of Brewer's spent grain showed that it contained a considerable amount of carbohydrate (6.4 %) and protein (20 %) that could be a good source for microorganism's growth and metabolic activities. Effect of different supplements was studied on solid state fermentation of Brewers' spent grain. Among the different constituents, the organic nitrogen that is peptone was found to give significant results at 0.1 % concentration which increases the citric acid production up to 0.19 % as compared to yeast extract which did not give significant result in the fermentation medium. The effect of inorganic constituents on the fermentation showed that potassium dihydrogen phosphate was also found best at 0.1 % which led to increase in the production of citric acid i.e (0.22 %). The mineral constituents such as ammonium sulphate and magnesium sulphate were found effective at 0.1 % concentration. From this investigation, it can be concluded that BSG can be used as a cheap raw material for the production of citric acid by *A. niger.*

Keywords: *Aspergillus niger*, Brewers' spent grain (BSG), Citric acid, Solid state fermentation.

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Introduction

Citric acid is one of the most important organic acids used in the food, beverage, pharmaceutical, chemical, cosmetic and other industries. It is used as acidulant, antioxidant, flavour enhancer, preservative, and as a synergistic agent[1]. Being a tricarboxylic acid with a molecular weight of 192 g/mole (anhydrous) and 210 g/mole (monohydrate), its production can be undertaken by different groups of microorganisms. Although yeast, bacteria and other *Aspergillus* species can produce citric acid, *Aspergillus niger* remained the organism of choice for commercial production, because it produces more citric acid[2]**.**

The production of citric acid using cheap carbon source from agro-industrial by-products provides a considerable advantage over the benefit of waste management as well as a decrease in the cost of citric acid production[3,4]. To date, most industrial processes are carried out with submerged fermentation of *Aspergillus niger*. It has been used commercially for the first time in 1923 for citric acid production. In order to decrease the cost of citric acid production using *A. niger*, solid state fermentation has been studied as a potential alternative to submerged fermentation.

Waste utilization or disposal in food industries is a major problem in maintaining sanitation and avoiding pollution of land, air and water[5]. Thus, brewer's spent grain is a readily available, high volume low-cost by-product of brewing and is a potentially valuable resource for industrial exploitation[6]. The recent increase in pollution load on earth has led to the development of processes that can reduce the environmental issues. Based on this, the potential of BSG as a substrate for production of citric acid by solid state fermentation (SSF) was evaluated and the fermentation parameters for utilization of this waste were optimized.

Materials and Methods

Microbial culture, spore suspension and brewer's spent grains

The *Aspergillus niger* strain MTCC 281 was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology,

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Chandigarh. Further, the culture was sub-cultured on potato dextrose agar (PDA) slants and potato dextrose broth (PDB) and stored at 4 ºC. The inoculum was prepared by suspending one loop of inoculating needle (spores of 5-day-old culture) in 1 mL of normal saline (0.2 % in 25 mL) and shaken vigorously. The spent grains were procured from Brewery, Distt. Solan, H.P. The collected spent grains were dehydrated in the mechanical dryer at 60 ºC for 2-3 hours and the proximate analysis for chemical composition of spent grains was done for moisture content, crude fibre, crude protein, ash content, total sugars, reducing sugars and titratable acidity.

Fermentation medium

The fermentations were performed in four 250 mL conical flasks containing 150 mL of the fermentation medium (g/L): peptone, 2; yeast extract, 1.5; potassium dihydrogen phosphate, 2; magnesium sulphate, 2; ammonium sulphate, 2 and brewers spent grain (30 g), containing water 1:5 ratio. The initial pH was adjusted to 5.5 with 0.1 M NaOH or HCl. Before inoculating, the flasks were autoclaved at 121 ºC for 20 mins.

Optimization of fermentation medium constituents for citric acid production

Different combinations of each medium constituent were tried for citric acid production by SSF. The experiments were conducted in three replications in four 250 mL Erlenmeyer flasks containing 30 g of the substrate with 150 mL fermentation medium maintaining 1:5 dilution in each and every experiment. Each medium constituent with its different concentrations was compared with each other to get effective concentration for citric acid production. The titratable acidity and pH were measured on alternate days and reducing sugar content was observed at the end of fermentation. The results were analyzed on the basis of the mean of values of all experiments conducted in three replications.

Effect of different concentrations of peptone, yeast extract, potassium dihydrogen phosphate, ammonium sulphate and magnesium sulphate on citric acid production

Different medium constituents were used for production of citric acid. These were used in following concentration i.e. Peptone (0.3 g); Yeast extract (0.225 g); KH_2PO_4 (0.3 g); $(NH_4)_2SO_4$ (0.3 g); $MgSO_4$ (0.3 g). To get effective concentration for citric acid production different concentrations of medium constituent were prepared. In each combination, one ingredient was varied and other ingredients were kept constant e.g. different concentrations of Peptone [Control (without peptone), 0.1 % (0.15 g); 0.2 % (0.3 g); 0.3 % (0.45 g)] were added to four different 250 mL Erlenmeyer flasks containing 30 g spent grains with 150 mL medium (YE, 0.225 g; KH_2PO_4, 0.3 g; $(NH_4)_2SO_4$, 0.3 g; $MgSO_4$, 0.3g) . Likewise different concentrations of Yeast extract [Control (without Yeast extract), 0.1 % (0.15 g), 0.2 % (0.225 g), 0.3 % (0.3 g)]; Potassium dihydrogen phosphate [Control (without KH_2PO_4), 0.1 % (0.15 g), 0.2 % (0.3 g), 0.3 % (0.45 g)]; Ammonium sulphate [Control (without $(NH_4)_2SO_4$), 0.1 % (0.15 g), 0.2 % (0.3 g) , 0.3 % (0.45 g)]; Magnesium sulphate [Control ($MgSO_4$), 0.1 % (0.15 g), 0.2 % (0.3 g), 0.3 % (0.45 g)] were added to four different 250 mL Erlenmeyer flasks containing 30 g spent grains with 150 mL medium of other constituents.

Analytical methods

The proximate analysis of BSG was done for its chemical evaluation that included moisture content, ash content, crude fiber[7], pH, crude protein[8], titratable acidity[9], reducing sugars, total sugars[7]. The pH was taken with Wensar, WHP-10 pH meter, after calibrating it with buffer solutions of pH 4 and 9.223. Titratable acidity was estimated by treating a known aliquot of the sample against N/10 NaOH solution using phenolphthalein as an indicator. The titratable acidity was calculated and expressed as percent citric acid[9]. Determination of reducing sugars was based on DNS method[8]. The colour was compared with a set of standards (glucose, 10-100 µg) in a spectrophotometer-20D, at 510 nm.

Results and Discussion

The chemical composition of the Brewer's spent grain is shown in Table 1. A gradual increase in citric acid production up to certain period was observed throughout the fermentation period. The reducing sugar content and pH correlates with the rate of citric acid production. Fermentation flasks of treatment containing 2 % peptone produced the highest concentration of citric acid content (Table 2). Results shown in Table 3 reveals that on the addition of yeast extract, there was no significant contribution towards citric acid accumulation. Further, results presented in Tables 4-6 showed that the addition of mineral constituents was equally necessary for the citric acid

Table 1 — Chemical characteristics of Brewer's spent grains

Characteristics	Mean±SD
Moisture Content (%)	8.94±0.23
Total sugars (%)	6.4±0.16
Reducing Sugars (%)	1.7±0.16
Titratable Acidity (% Citric acid)	0.064±0.08
Protein (%)	20±0.31
Ash Content (%)	3.75±0.04
Crude fibre	3.4±0.1

Data are presented as mean±SD (n= 3)

Table 2 — Effect of Peptone addition on Solid state fermentation of Brewer's spent grains

Treatment	Peptone Concentration (%)	Titratable Acidity (% Citric acid)	pH	Reducing Sugars (%)
T_1 (Control)	0	0.15	2.8	0.3
T_2	0.1	0.19	2.2	0.23
T_3	0.2	0.16	2.4	0.26
T_4	0.3	0.15	2.7	0.28

Table 3 — Effect of Yeast Extract addition on Solid State Fermentation of Brewer's Spent Grains

Treatments	Yeast extract Concentration (%)	Titratable acidity (% Citric acid)	pH	Reducing Sugars (%)
T_1 (Control)	0	0.22	2.4	0.23
T_2	0.1	0.15	3.5	0.24
T_3	0.2	0.12	3.7	0.26
T_4	0.3	0.10	4.0	0.28

Table 4 — Effect of potassium dihydrogen phosphate addition on Solid state fermentation of Brewer's spent grains

Treatments	KH_2PO_4 Concentration (%)	Titratable acidity (% Citric acid)	pH	Reducing sugars (%)
T_1 (Control)	0	0.12	2.8	0.27
T_2	0.1	0.18	2.3	0.22
T_3	0.2	0.14	2.5	0.25
T_4	0.3	0.06	2.9	0.3

yield. The addition of 1 % potassium dihydrogen phosphate in Table 4 showed a significant deviation for citric acid production with respect to other concentrations. The concentrations of 1 % of both ammonium sulphate and magnesium sulphate were found to be the best for citric acid production as shown in Tables 5 and 6.

In this investigation, optimization and effect of different medium constituents was studied on solid state fermentation of BSG for citric acid production

Table 5 — Effect of ammonium sulphate addition on solid state fermentation of Brewer's spent grains

Treatments	$(NH_4)_2SO_4$ Concentration (%)	Titratable Acidity (% citric acid)	pH	Reducing sugars (%)
T_1 (Control)	0	0.14	3.3	0.3
T_2	0.1	0.19	2.4	0.23
T_3	0.2	0.17	2.7	0.26
T_4	0.3	0.15	3.0	0.28

Table 6 — Effect of magnesium sulphate addition on solid state fermentation of Brewer's spent grains

Treatment	$MgSO_4$ concentration (%)	Titratable acidity (% Citric acid)	pH	Reducing sugars (%)
T_1 (Control)	0	0.20	2.6	0.27
T_2	0.1	0.23	2.4	0.24
T_3	0.2	0.19	2.7	0.35
T_4	0.3	0.15	2.9	0.42

using *A. niger*. The production of citric acid continued an increased up to a particular day after which a decline trend was observed in all the experiments. Peptone is one of the most essential constituent of a nutrient medium for fungi. Soccol *et al*[2] reported that the increase in peptone concentration adversely affected the yield of citric acid production and reducing sugars were more consumed. The more the acid, the lower would be the pH. The results are in confirmation with the earlier report that high nitrogen concentrations increase fungal growth and sugar consumption but decrease the amount of citric acid produced. The reducing sugar content correlated with pH. It is clearly evident from Table 2 and 3 that in comparison to peptone, yeast extract showed no significant increase in citric acid production. It might be due to the reason that the micro organism preferred peptone as a good source of nitrogen because each nitrogen source has different effect on the development of fungus and metabolite production[11]. Jennison *et al.*[12] have reported that intrinsic differences in the molecular structure may be involved in the differences in utilization of these nitrogen compounds. The concentration of phosphate in the fermentation medium is also very important for the production of citric acid. Shu and Johnson[13] reported that the phosphate ions are essential for citric acid production in addition to their effect as a buffer and food constituent. The maximum yield of citric acid, obtained in the case of KH_2PO_4 and K_2HPO_4 indicates that K^+ ion has also a role together with phosphorus

in citric acid fermentation. It was also reported that the presence of excess of phosphate leads to a decrease in the fixation of carbon dioxide, which in turn increases the formation of certain sugar acids, and the stimulation of growth[14,15,2]. This can be related to the fact reported in literature earlier that acid ammonium compounds are preferred because their consumption leads to pH decrease, which is essential for the citric fermentation[16]. High nitrogen concentrations increase fungal growth and sugar consumption but decrease the amount of citric acid production[2]. Our results are in conformation with the earlier reports showing that magnesium probably takes part in the activation of enzymes necessary for the normal growth and metabolism[17,18]. So, the concentration of $MgSO_4$ should be varied appreciably as trace metal ions have a significant impact on citric acid accumulation by *A. niger*[19].

Further, the results are in accordance with the earlier results of Karow[20] that the amount of magnesium sulphate used may be varied appreciably without seriously affecting the growth or citric acid formation, as long as sufficient amount is furnished to supply the basal requirement. It appears that citric acid production is linked to the utilization of carbon by *A. niger*. In all the experiments, the citric acid production followed an exponential trend. As the production continued, the content of sugars and pH decreased. The complete exhaustion of sugars led to the decline in production of citric acid. The concentrations of particular nutrient should be varied considerably to get effective production of citric acid. The optimum level of concentration can lead to higher yields of citric acid, below and beyond which it adversely affect the production. There are reports of the use of different optimization techniques to improve citric acid production by *A. niger* in both submerged and solid state fermentation[21-23].

Conclusion

Increasing demand for citric acid in various applications has led to the ways to be explored for its efficient production. Solid state fermentation is a way to increase the productivity of citric acid. The use of agro-industrial wastes as a substrate for citric acid production certainly helps in the reduction of production costs associated with costly synthetic substrates. In brief, the use of nitrogen and mineral sources not only differs from species to species but also from strain to strain. The concentration of a particular nutrient should be well optimized should be varied appreciably because beyond optimize concentration it was adverse effects on production rate were observed further it can be concluded that brewer's spent grains hold promise for production of value-added products at still cheaper cost and in an eco-friendly way resulting in minimizing environmental issues. So, a proper modelling of solid state fermentation and standardization of conditions are to be taken into consideration for effective citric acid production from brewer's spent grains.

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