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CITRIC ACID PRODUCTION BY *Aspergillus niger* REPEAT-BATCH CULTURE USING A PRICKLY PEAR CACTUS EXTRACT MEDIUM

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ABSTRACT

The potential of prickly pear cactus extract as a fermentation medium for citric acid production by batch and repeat-batch cultures of *Aspergillus niger* isolated from three locations of Aseer, Saudi Arabia was investigated. Among the 23 isolated cultures of *Aspergillus niger*, the isolate J6 which obtained from Jazan was found to be enhance citric acid production. Maximum citric acid production (61.56 g/L) was obtained after 8 days of cultivation with an initial pH 4 and 20 % sugar concentration. Better results were achieved when applying repeat-batch culture than batch cultivation. The culture was retained their activity for 24 days of fermentation period and the concentration of citric acid was remained constant during recycle of biomass up to the third batch. A total amount of citric acid of 244.75 g was obtained after five repeated batch-cultivation. On the basis of the experimental results, it was suggested that the prickly pear cactus extract can serve as a low-cost fermentation medium for citric acid production using *Aspergillus nigar* repeat batch-culture.

INTRODUCTION

Prickly pear cactus (*Opuntia ficus-indica*) was widely distributed in Saudi Arabia, either as a cultivated plant for human food or as hedge plant (Collenette 1999). Its survival in dry regions was associated with their ability to store water in the thick succulent stem and obtain this water through widely, spreading and shallow root system (Everard and Morley 1976). The pulp of prickly pear cactus was found to be rich in carbohydrates (glucose 35% and fructose 29%) and protein (5.1%), while its crude lipids and fibers contents were found to be quite low (El-Kossori *etal.*, 1998). The utilization of cactus pear fruits was studied by many investigators such as Oliveira (2001), who used the natural polyelectrolytes, extracted from cactus as auxiliary of flocculation and coagulation in wastewater treatment and for microbial protein production, while Saenz *et al.*, (1988) and Fernandez-Lopez and Almela (2001), used the fruits to obtain a new natural liquid sweetener and two pigments, indicaxanthin and betanin respectively. However, prickly pear cactus was a neglected nutritional source as a medium for citric acid production. Citric acid was a broadly used in the pharmaceutical, food and beverage industries (Kundu *etal.*, 1984; Roukas and Alichanidis 1991) . The current world market estimates suggest that upwards of 4.0×10^5 tones citric acid per year may be produced (Kristiansen *etal.*, 1999). Surface and submerged batch culture techniques are still being used for citric acid production, however, in the batch technique a significant amount of productive biomass was discarded each time (Sing *etal.*, 1998; Pazouki *etal.*, 2000). Repeat-batch culture, allows maintenance of more stable activity of

the culture for a long period and decrease the expenditure of sterilization and preparation of inoculums and fermentor which increase the economical efficiency of citric acid production (Arzumanov *et al.*, 2000). Because of the widely distribution of prickly pear cactus in Saudi Arabia and its high concentration of soluble sugars, the aim of this study was to make the extract of prickly pear cactus suitable to be use for citric acid production by *Aspergillus nigar* isolated from Aseer region using repeat -batch culture technique.

MATERIALS AND METHODS

Microorganism and screening of isolated cultures:

Wild strains of *Aspergillus nigar* were isolated from soil samples collected from three different locations of Aseer, (Abha, Jazan and Najran) Saudi Arabia, and maintained in PDA slant agar at 5 C°. The identification was made according to Roper and Fennell (1965). These cultures were screened for citric acid production by its cultivated in a shaker incubator for 6 day at 30 C° and 150 r.p.m using a prickly pear cactus extract as a growth and production medium (pH, 5).

Inoculum:

The cultures were incubated on PDA slant at 30 C° for 5 days. The spores were suspended in 5 ml sterile distilled water to prepare the inocula.

Fermentation medium:

The prickly pear cactus was cut in small pieces and 132g of cactus pieces with 750 ml of tap water were transferred to a 2 L flask with stirring for 30 min. The extraction of viscous natural polyelectrolytes (soluble sugar) was performed by maceration. The extract (fermentation medium) was concentrated at 50 C° under vacuum to contain 20 % initial sugar (Oliveira 2001).

Repeat-batch cultivation:

For the first batch, a set of Erlenmeyer flasks (250-ml) containing 100 ml fermentation medium (pH, 5) were inoculated with 1 ml of the inoculum to give a final concentration of $2x10^7$ spores / ml. The cultures were kept in a shaking incubator at 30 C° and 150 r.p.m for different periods up to 12 days. When the fermentation was complete, the original medium was withdrawn from each flask and replaced with 100 ml of fresh sterile fermentation medium (Arzumanov *etal.*, 2000).

Analytical methods:

At the end of each batch cycle, the fermentation liquid was used for the calorimetrically determination of residual sugars by phenol sulfuric acid method (Duboise 1956). Anhydrous citric acid was estimated by pyridine-acetic anhydride method (Marrier and Boulet 1958) using a double beam UV/Vis scanning spectrophotometer (Model: Shimadzu, 1601PC). Culture dry weight was determined according to Haq and Daud (1995).

RESULTS AND DISCUSSIONS

Screening of *Aspergillus niger* isolates for citric acid production on a prickly pear cactus extract medium:

Twenty three isolates of *Aspergillus nigar* (Table 1) were obtained from soil samples of three localities of Aseer, Saudi Arabia, 7 of which were isolated from Abha, 11 isolated from Jazan and 5 isolated from Najran. The frequencies of isolates in the examined localities may be affected by the nature and component of the soil, plant activates and prevailing environmental conditions as reported by Hashem and Al- Faraj (1995). These isolates were screened for citric acid production on a pear cactus extract as a fermentation medium without supplements. The results presented in Table (1) indicated that all the tested isolates were able to produce citric acid on a pear cactus medium. Of these culture, *Aspergillus nigar* j6 isolated from Jazan was produced the highest amount of citric acid (41.71g/L) followed by isolate j6 from the same region (33.52 g/L). On the other hand, a large differences in the citric acid production by isolated cultures were also recorded. Isolates j4, j8 and j10 from Jazan and A4 and A5 from Abha were capable to produce favorable amount of citric acid, while slightly amounts were observed with isolates N3 and N5 from Najran and A2 and A6 from Abha. Highest citric acid obtained was not only dependent upon isolated organisms, but also the beneficial characters of pear cactus extract as a fermentation medium. These results were in harmony with the findings of Aravantinos-Zafiris *etal.,* (1993) and Sikander *etal.,* (2002). *Aspergillus nigar* j6 isolated from Jazan was selected as a good producer for citric acid in the following experiments.

Table (1): Screening of *Aspergillus nigar* isolates for citric acid production on a prickly pear cactus extract medium

Locality	*Aspergillus nigar* isolates	Citric acid g /L
Abha	A1	11.7
	A2	5.7
	A3	9.2
	A4	22.6
	A5	18.7
	A6	7.1
	A7	13.8
Jazan	J1	16.43
	J2	9.70
	J3	13.80
	J4	23.15
	J5	11.56
	J6	41.71
	J7	21.15
	J8	17.8
	J9	33.52
	J10	25.11
	J11	13.4
Najran	N1	10.6
	N2	16.5
	N3	4.5
	N4	12.1
	N5	8.71

Effect of fermentation period:

The results illustrated in Fig (1) revealed that the production of citric acid by *Aspergillus nigar* j6 was started after a lag phase of two days of cultivation and increased parallel with fungal biomass as fermentation progressed up to 8 days reached to 48.98 g/L at the late of stationary phase. These results were confirmed by Rohr *etal.,* (1983) and Sikander *etal.,* (2002), who reported that citric acid fermentation was considered type II fermentation, this type was characterized by two phases, growth and production phases. At the optimum fermentation period, biomass and sugar utilization were 17.81 g/L and 87.58 % respectively. Further increasing of fermentation period did not enhance citric acid production, because this will reduce the amount of nitrogen available in fermentation medium, depletions of sugar contents and the decay in enzyme system responsible for biosynthesis of citric acid (Kristiansen and Sinclair 1979; Berovic *etal.,* 1991; Alvarez-Vasquez *etal.,* 2000 and Arzumanov *etal.,* 2000).

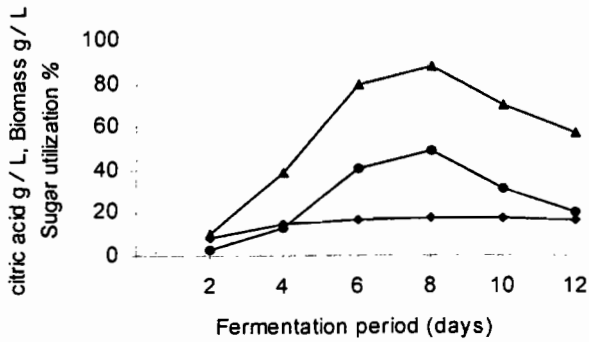

Fig (1): Effect of fermentation period on citric acid production. Citric acid (•), Sugar utilization (▲), Biomass (♦).

Effect of initial pH:

The initial hydrogen ion concentration of fermentation medium was one of the critical factors that have a profound effect on the citric acid production. The pH profile of citric acid synthesis was illustrated in Fig (2). The optimum citric acid production was obtained at pH 4 reached to 61.76 g/L with increasing the sugar utilization to 92.45 %. Biosynthesis of citric acid was decreased at higher pH reached to 18.54 g/L at pH 7 and lost about 88% if pH value was changed to pH 8. These observation were an agreement with Sikander *etal.*, (2002), who reported that the highest initial pH leads to the accumulation of by products such as oxalic acid. Different investigators were also used pH value around 4 as the optimum pH for citric acid production. (Pessoa *etal.*, 1982; Arzumanov *etal.,* 2000; Bayraktar and Mehmetoglu 2000) .

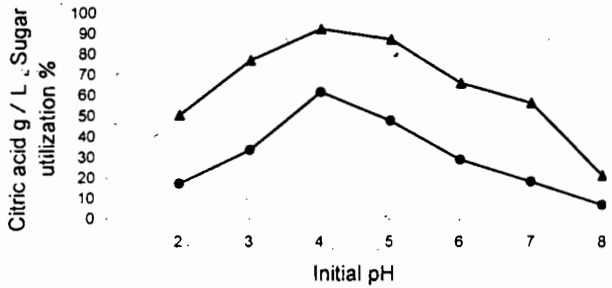

Fig (2): Effect of initial pH on citric acid production. Citric acid (•), Sugar utilization (▲).

Effect of sugar concentration:

The effect of sugar concentration on citric acid production was examined with media containing 10,15,20,25,30 %. The results illustrated in Fig (3) revealed that the maximum value of citric acid was produced at 20 % sugar with sugar utilization of 92.30 % . Any increase or decrease other than this value, resulted in the disturbance in biosynthesis of citric acid. Production of citric acid was reduced to 68.12 % at 10 % sugar and 44 % at 30 % sugar concentration. The advantages of high fermentable sugars concentration in the fermentation medium may be due to the reduced dilution water requirements and suppression of osmosensitive contaminates (Roukas 1998).

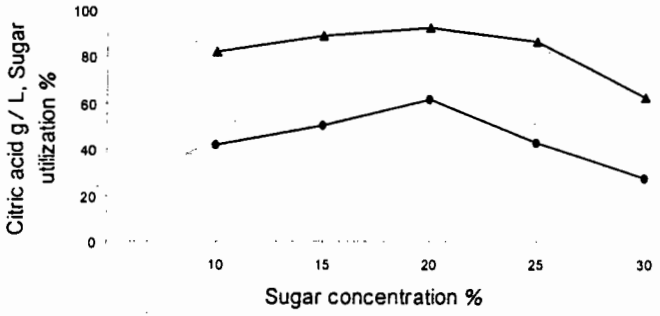

Fig (3): Effect of sugar concentration on citric acid production. Citric acid (•), Sugar utilization (▲).

Citric acid production by Repeat-batch cultivation:

As mentioned previously, the fermentation was conducted in batch mode and then the culture liquid was withdrawn and fresh fermentation medium was added. By reuse the biomass, five batches of cultivation were carried out in total. The results illustrated in Fig (4: A-E) indicated that repeat-batch culture method achieves better results than batch cultivation. Time needed for maximal citric acid production was differed among the batches. At the first three batches, citric acid concentration was reached to maximal level after 8 days, while optimum fermentation period for two late batches were 6

days. The results revealed to the culture of *Aspergillus nigar* was retained their activity for 24 days and the citric acid production was remained almost constant during recycle of biomass up to third batch and then sharply decreased. These finding may be due to the enzymes involved in citric acid biosynthesis remained stable over time in repeat-batch culture compared with batch cultivation (Berovic *etal.*, 1991; Arzumanov *etal.*, 2000) . The low citric acid observed after three batches may be due to the high biomass concentration in the fermentation medium and a large amount of sugar consumed were converted to biomass instead of citric acid production as reported by Roukas (1998). A total amount of citric acid of 244.75 g were reached after five repeated- batch cultivation. These results were in line with those of Roukas (1998) and Vassileva *etal.*, (1998). Finally, the results demonstrated that the prickly pear cactus can serve as a low-cost medium for citric acid production using repeat-batch cultivation up to three times that could result in substantial cost savings.

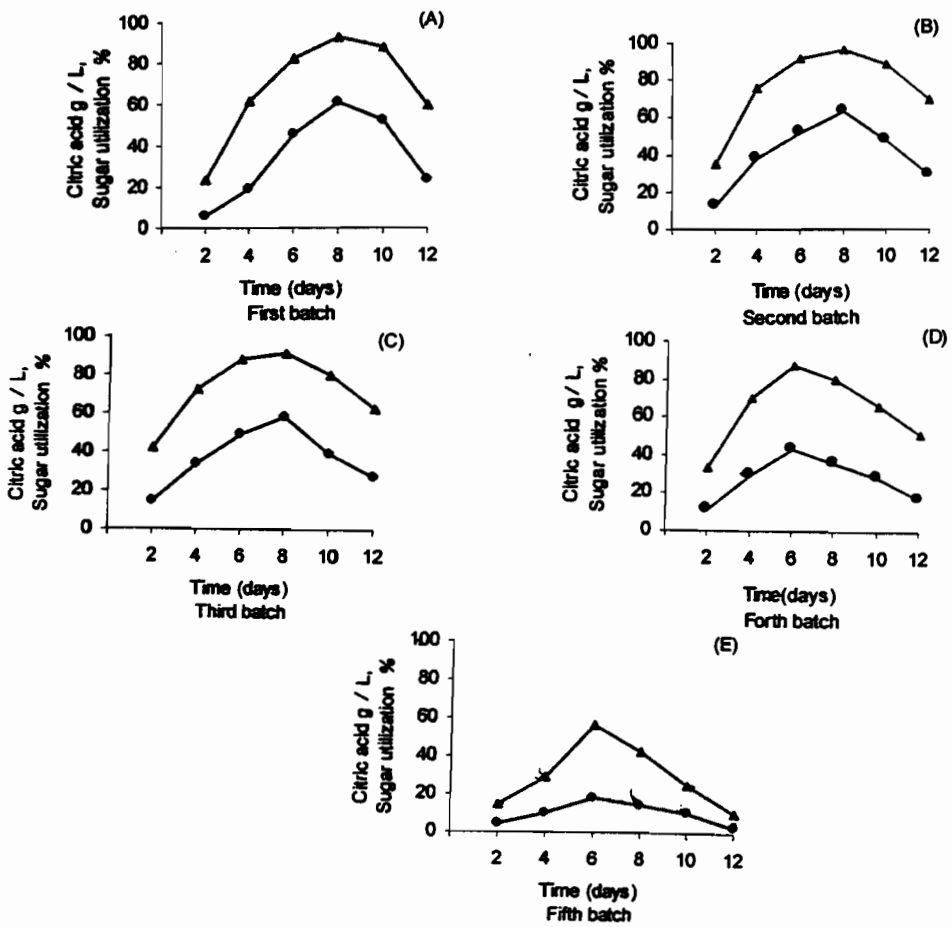

Fig (4: A-E) : Citric acid production by *Aspegillus nigar* repeat – batch culture. Citric acid (•), Sugar utilization (▲).

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إنتاج حامض الستريك بواسطة مزارع الأسبرجلس نيجر ذات الدفعة المتكررة باستخدام بيئة مستخلص التين الشوكي

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تهدف هذة الدراسة إلى الوقوف على إمكانية استخدام مستخلص التين الشوكي كبيئة لإنتاج حامض الستريك حيث ينتشر بصورة كبيرة في منطقة عسير بالمملكة العربية السعودية علاوة على أنة يعد مصدر إقتصادى رخيص كمادة أولية للتخمرات الميكروبية باستخدام تكنيك مزارع الدفعة الواحدة و مزارع الدفعة المتكررة. تم عزل ٢٣ عزلة تتبع فطر الأسبرجلس نيجر من ثلاث مواقع من منطقة عسير و اختبرت لمعرفة قدرتها على إنتاج الحامض و أسفرت النتائج على اختيار العزلة j6 المعزولة من منطقة جازان كأحسن عزلة لإنتاج الحامض على بيئة مستخلص التين الشوكي. أعلى إنتاج للحامض تم الحصول علية بعد ٨ أيام من التنمية في ظل درجة أس هيدروجيني ٤ و تركيز سكر ٢٠ %. أظهرت النتائج أيضاً أفضلية كبيرة لتكنيك مزارع الدفعة المتكررة عن الدفعة الواحدة حيث احتفظت المزرعة بثبات في الإنتاج أمتد لثلاث دفعات متتالية بإجمالي ٢٤ يوم ثم انخفض الإنتاج بعد ذلك بشكل ملحوظ . أختلف أيضاً الوقت المثالي للإنتاج باختلاف الدفعات فكان أعلى إنتاج للحامض بعد ٨ أيام من التنمية خلال الثلاث دفعات الأولى بينما كان أفضل إنتاج للحامض خلال أخر دفعتين عند ٦ أيام. من النتائج المتحصل عليها يمكن استنتاج إمكانية استخدام مستخلص التين الشوكي كبيئة اقتصادية لإنتاج حامض الستريك باستخدام تكنيك المزارع ذات الدفعة المتكررة حتى ثلاث دفعات.