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Gene Transfer Between Different *Trichoderma* Species and *Aspergillus Niger* Through Intergeneric Protoplast Fusion to Convert Ground Rice Straw to Citric Acid and Cellulases

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Abstract

Single-stage direct bioconversion of cellulosic materials to citric acid using intergeneric hybrids obtained from three different *Trichoderma* species and *Aspergillus niger* was carried out. The recent results were obtained on the basis of either resistance or sensitivity to one or more of five metal ions, two catabolite repressors, and five antifungal agents, which were used in this study at different concentrations. Sixty-six fusants were isolated after using the three intergeneric protoplast fusion experiments, belonging to two types of intergeneric fusants. Fusants of the first type are heterokaryons (35 fusants). On the other hand, those of the second type are haploids (31 fusants), i.e., they were stable. The present study can be successfully applied in the construction of 14 new genetic fusants, which produced at least 100% more citric acid than the citric acid producer strain *A. niger*. Out of the fusants, three (1/18, 2/13 and 2/15) showed about a threefold increase of citric acid production in comparison with the parent *A. niger* strain. Furthermore, studies on DNA content showed that this finding may be submitted on the evidence that citric acid and cellulases production was not correlated with DNA content; however, the productivity depends on specific DNA content.

Index Entries: Citric acid; cellulases; protoplast fusion; *Trichoderma* spp.; *Aspergillus niger*.

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Introduction

Citric acid is one of the commodity chemicals that is widely used in food, pharmaceutical, cosmetic, textile and beverage industries. It is primarily produced from molasses or starch hydrolysate using the filamentous fungus *Aspergillus niger (1,2)*. It is important to establish production processes from inexpensive and readily available raw materials. Agricultural waste, in the form of plant biomass containing a large amount of cellulosic materials, is underutilized. However, direct production of citric acid from cellulose has not yet been successful because the citric acid-producing strain *A. niger*, does not degrade crystalline cellulose nor assimilate it as a sole carbon source.

In the field of biotechnology, the improvement of different strains of fungi is important because they participate in the core activities of many industries. The technique of protoplast fusion is used as a bypass from the normal mating of cells and is thus a powerful tool in the improvement of microorganisms *(3)*. Pham and Saturnina *(4)* found out that viable protoplast fusants can be derived from *Trichoderma reesei* RUT C-30 and *Penicillium funiculosum* Thom MG-171, which may exhibit improved characteristics when compared to the parents.

Protoplast fusion is widely used in genetic engineering. This method offers new prospects for obtaining microorganisms displaying new properties *(5,6)*. Commonly, the strategy applied to protoplast fusion relies on either the forced nutritional complementation of auxotrophic mutant strains or resistance to fungicides of parental strains *(7,8)*. Kirimura et al. *(9)* carried out intergeneric protoplast fusion between *A. niger* (producing citric acid) and *Trichoderma viride* (producing cellulases) and have succeeded in obtaining two types of intergeneric fusants, the first were haploids whereas the second were heterokaryons. However, the direct bioconversion of cellulosic materials to ethanol by the intergeneric fusant between *T. reesei* and *Saccharomyces cerevisiae* appears to be one of the best techniques for an alternative process for ethanol production *(10)*. In this study three different *Trichoderma* species (producing cellulases) were used to be fused with *A. niger* (producing citric acid) in a trial aiming to produce citric acid in one stage from ground rice straw.

Materials and Methods

Fungal Strains

The microorganisms used in this work were the three *Trichoderma* species; *T. reesei* NRRL 18670, *Trichoderma harzianum* NRRL 13879, and *T. viride* strain as cellulases producers and *A. niger* NRRL 599 as citric acid producer. *T. viride* was obtained from Microbiology Department, Faculty of Agriculture, Mansoura University, Egypt and the other strains from the United States Department of Agriculture, Agriculture Research Service, National Center for Agricultural, Utilization Research.

Media and Conditions

Cultures were maintained by reinoculating on malt extract agar (Merck). The composition of the protoplast medium was (g/L in distilled water): glucose, 80.0; NH_4NO_3, 2.0; KH_2PO_4, 10.0; $MgSO_4 \cdot 7H_2O$, 0.25; $FeCl_3 \cdot 6H_2O$, 0.02; $MnSO_4$, 0.14 and the initial pH of the medium was adjusted to 4.25. Fifty milliliters of medium were dispensed into 250-mL Erlenmeyer flask for the development of mycelium. The flasks were incubated at 30°C for 20 h on a shaker maintained at 160 rpm *(11)*.

Hypertonic, selective, and regeneration medium is the same as the protoplast medium with the addition of 10.0 g/L cellulose as a carbon source instead of glucose, 0.7 *M* KCl, 0.1% (v/v) Triton X-100, 20.0 g/L agar, and one or more of the antifungal agents, heavy metal ions, and/or catabolite repressors.

The composition of modified fermentation medium for cellulases and citric acid production was (g/L in tap water): ground rice straw, 30.0; cellulose, 1.0; bactopeptone, 5.0; KH_2PO_4, 5.0; yeast extract, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5, and the pH of the medium was adjusted to 7.0. Citric acid and cellulases production by the shaking culture was carried out in 250-mL Erlenmeyer flasks containing 50-mL fermentation medium. The medium was inoculated with 5 mL of the spore suspensions from eight days old slants with distilled water containing a drop of Tween-80. Flasks were incubated with shaking (200 rpm) at 30°C for 10 d.

Screening for Metal Ions Resistance

To establish resistance, the metal resistance of the parental strains during growth on hypertonic regeneration medium was studied with the use of Co^{++}, Cu^{++}, Fe^{+++}, and Zn^{++} metal ions at different concentrations, i.e., 500 and 1000 ppm, whereas the Hg^{++} metal ion was used at 200 and 300 ppm. Resistance or sensitivity to the metal ions was used as a marker. These complementary markers were used for the fusion treatments.

Catabolite Repressors Resistance

Different concentrations, 0.1 and 0.2% (w/v) from 2- deoxyglucose and 15 and 20% (v/v) from glycerol, were added to hypertonic regeneration medium and used as catabolite repressors to mark the parental strains. These complementary markers were used for the fusion treatments.

Response to Antifungal Agents

For the isolation of antifungal resistant strains, hypertonic regeneration medium and five antifungal agents were used, concentrations of antifungal agents added were as follows: 1.0 and 2.0 µg/mL benomyl, 1.0 and 2.0 µg/mL miconazole, 400 and 500 µg/mL griseofulvin, 100 and 250 µg/mL cycloheximide, and 10 and 25 µg/mL 5-fluorouracil. Colonies that exhibited resistance or sensitivity to a specific antifungal were retested on the

same antifungal dose to be sure of their resistance or sensitivity stability and used as markers to select the fusants.

Protoplast Formation

Protoplasts were prepared through enzymatic hydrolysis of mycelium suspension using the procedure of EL-Bondkly *(12)*. Cultures were grown in liquid protoplast medium. After incubation, the mycelium was collected by centrifugation, washed twice with 0.7 *M* KCl in 25 m*M* phosphate buffer, pH 5.8, and then resuspended in (50 mg/mL) phosphate buffer containing 0.7 *M* KCl and 10 mg/mL Novozyme 234. The lytic mixtures were incubated at 30°C with gentle shaking for 3 h. Incubation mixtures were filtered and protoplasts were counted in the filtered lysate.

Intergeneric Protoplast Fusion

Protoplasts prepared from each of the two strains were mixed and suspended in 30% (w/v) polyethylene glycol solution containing 0.05 *M* glycin–NaOH buffer, pH 7.5, and 0.05 *M* $CaCl_2$. After incubation at 30°C for 10 min, the suspension was centrifuged at 2550*g* for 5 min. Treated protoplast pellets were resuspended in 1 mL of osmatically balanced phosphate buffer, diluted appropriately, and were plated on the hypertonic selective and nonselective regeneration media. Plates were incubated at 28°C until colonies were grown on the surface of plates. The grown colonies were considered as complementary fusants. They were transplanted and subcultured several times onto selective and nonselective media before further studies. Fusion frequency was expressed as the ratio of the number of colonies formed on selective and nonselective media.

Analytical Methods

Citric acid was estimated by the acetic anhydride and pyridine method of Marrier and Boulet *(13)*, also, the quantity of citric acid to high production fusants was examined by high-performance liquid chromatography (HPLC) analysis, an HPLC unit of Gilson**.** DNA was extracted from mycelia according to the method of Herbert et al. *(14)* using perchloric acid and measured by the method of Ceriotti *(15)* using indole. On the other hand, enzyme activities were measured on filter paper, carboxymethylcellulose and on *p*-nitrophenyl-β-D-glucopyranoside for exoglucanase, endoglucanase and β-glucosidase, respectively, as described by Mandels et al. *(16)*. The reducing sugars formed were measured by the DNS method *(17)*, whereas *p*-nitrophenol was measured using the method of Vaheri et al. *(18)*. One unit of the enzyme activity equals 1 µmol of glucose or *p*-nitrophenol produced per minute under the test conditions.

Results and Discussion

Because no one had any intention of altering the genetic make-up of the parental strains, this study sought for innate properties of these strains

to use as selection markers. In this work, the parental strains were screened to study their resistance to different metal ions (Hg^{++}, Co^{++}, Cu^{++}, Fe^{+++}, and Zn^{++}), catabolite repressors (2-deoxyglucose and glycerol), and antifungal agents (benomyl, miconazole, griseofulvin, cycloheximide and 5-fluorouracil) at different concentrations.

Metal Ions Resistance

The metal ions used for studying their effects on the parental strains (*T. reesei*, *T. harzianum*, *T. viride*, and *A. niger*) were grown on a medium containing either 200 or 300 ppm Hg^{++} or 500 and 1000 ppm from the other metal ions, Co^{++}, Cu^{++}, Fe^{+++}, and Zn^{++} were applied. Results in Table 1 showed that, *T. reesei* NRRL 18670 showed sensitivity to both concentrations of Hg^{++}, Co^{++}, Cu^{++}, and Fe^{+++} and resistance to both concentrations of Zn^{++}. On the other hand, *T. harzianum* NRRL 13879 was resistant to all concentrations of all metal ions used in this study. Furthermore, *T. viride* was resistant to all metal ions concentrations, except with 1000 ppm Cu^{++}. Whereas, *A. niger* NRRL 599 was resistant to 200 ppm of Hg^{++} and 500 ppm of Fe^{+++}, and sensitive to all concentrations of the other used metal ions.

Effect of Catabolite Repressors

Two different concentrations, 0.1 and 0.2% (w/v) from 2-deoxyglucose and 15 and 20% (v/v) from glycerol, were added to the medium and their effects were recorded. *T. harzianum* and *A. niger* showed resistance to all used concentrations of both 2-deoxyglucose and glycerol. On the other hand, *T. reesei* was sensitive to all different concentrations of catabolite repressors, except 0.1% (w/v) of 2-deoxyglucose. On the other hand, *T. viride* was resistant to all different concentrations of catabolite repressors except 0.2% (w/v) 2-deoxyglucose as well (Table 1).

Response of Parental Strains to Different Antifungal Agents

Table 1 presents the parental strains and their resistance or sensitivity to different concentrations of benomyl, miconazole, griseofulvin, cycloheximide, and 5-fluorouracil. Results showed that although the *T. reesei* was sensitive to all different concentrations of antifungal agents used, it showed, however, resistance to 10 µg/mL of 5-fluorouracil. On the other hand, *T. harzianum* was resistant to miconazole, griseofulvin, 5-fluorouracil, and cycloheximide at 100 µg/mL concentration. On the other hand, it was sensitive to 1.0 and 2.0 µg/mL benomyl and 250 µg/mL cycloheximide. *T. viride* was sensitive to benomyl, miconazole, and cycloheximide at all used concentrations; whereas, it showed resistance to griseofulvin and 5-fluorouracil at the used concentrations. The citric acid producer *A. niger* proved to be resistant to miconazole, griseofulvin, and cycloheximide at the used concentrations. In contrast, it proved to be sensitive to benomyl and 5-fluorouracil at the concentrations used. From the above results, it could be noticed that all the parental strains proved to be

Table 1
Response of Parental Strains to Different Metal Ions, Catabolite Repressors and Antifungal Agents at Different Concentrations

	Metal ions (ppm)										Catabolite repressors				Antifungal agents (µg/mL)									
	Hg^{++}		Co^{++}		Cu^{++}		Fe^{+++}		Zn^{++}		2-Deoxyglucose (w/v)		Glycerol (v/v)		Benomyl		Miconazole		Griseofulvin		Cyclo-heximide		5-Fluoro-uracil	
Strains	200	300	500	1000	500	1000	500	1000	500	1000	0.1%	0.2%	15%	20%	1.0	2.0	1.0	2.0	400	500	100	250	10	25
T. reesei NRRL 18670	–	–	–	–	–	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	+	–
T. harzianum NRRL 13879	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	–	+	+
T. viride	+	+	+	+	+	–	+	+	+	+	+	–	+	+	–	–	–	–	+	+	–	–	+	+
A. niger NRRL 599	+	–	–	–	–	–	+	–	–	–	+	+	+	+	–	–	+	+	+	+	+	+	–	–

+, resistance; –, sensitive.

sensitive to both concentrations used from benomyl. Different methods were applied for the detection of protoplast fusion. These methods included marker genes for drug resistance, auxotrophic mutants, morphological differences, catabolite repressors, as well as havey metal ions resistance. Resistance to the metal ions was also used as markers. These complementary markers were used for the fusion treatments. Cobalt and mercury resistant markers were introduced in *P. funiculosum* and *T. reesei*, respectively through induced mutations. The protoplasts of these fungi were fused and screened for hybrids as Co^R and Hg^R resistant colonies *(4,19)*. There were not recorded data for using catabolite repressors as selection markers in protoplast fusion, whereas many authors isolated *T. reesei* and *A. niger* mutants, which are 2-deoxyglucose resistant mutant strains *(2,20)*. Furthermore, drug resistance and antibiotic markers techniques have been used extensively in yeast species, particularly *S. cerevisiae (21)*. Meza et al. *(7)* selected fusants of *T. reesei* from 50 µg/mL benomyle—containing agar plates after three independent fusion experiments. On the other hand, EL-Bondkly *(12)* used five antifungal agents; nystatin, benomyle, griseofulvin, cycloheximide, and miconazole at different concentrations to label the parents before protoplast fusion.

Protoplast Formation

According to the conditions described under materials and methods, enzymatic treatments and subsequent examination of the treated mycelia from cellulases and citric acid producer parental strains with a phase-contrast microscope showed that gradual degradation of fungal mycelia started after the addition of 10 mg/mL Novozyme 234 enzyme. The whole cell wall digestion was achieved following incubation at 30°C with gentle shaking for 3 h as shown in Fig. 1. Maximum release of protoplasts was obtained with *T. reesei* NRRL 18670 that yielded 2.5×10^6 protoplasts per mL. From the mycelium of *T. harzianum* NRRL 13879 the highest yield of protoplasts, 3.5×10^6 was obtained after a 3-h incubation period. From the mycelium of *T. viride* the highest yield of protoplasts was 2.0×10^6 obtained after 3 h incubation time. On the other hand, 1.8×10^5/ mL protoplasts were released from *A. niger* NRRL 599 mycelium after a 3-h incubation time.

Intergeneric Protoplast Fusion and Regeneration

On the basis of the obtained results to either resistance or sensitivity to one or more of the five metal ions, two catabolite repressors, and five antifungal agents used in this study at different concentrations, three intergeneric protoplast fusion experiments were applied between three different *Trichoderma* spp. as cellulases producers and the citric acid producer *A. niger* strain.

Equal amounts of protoplasts prepared from young mycelia of each of the two strains were fused. Fusion frequency, which is the ratio of the number of colonies regenerating on the nonselective medium to the number of colonies formed on the selective medium, was found to be different

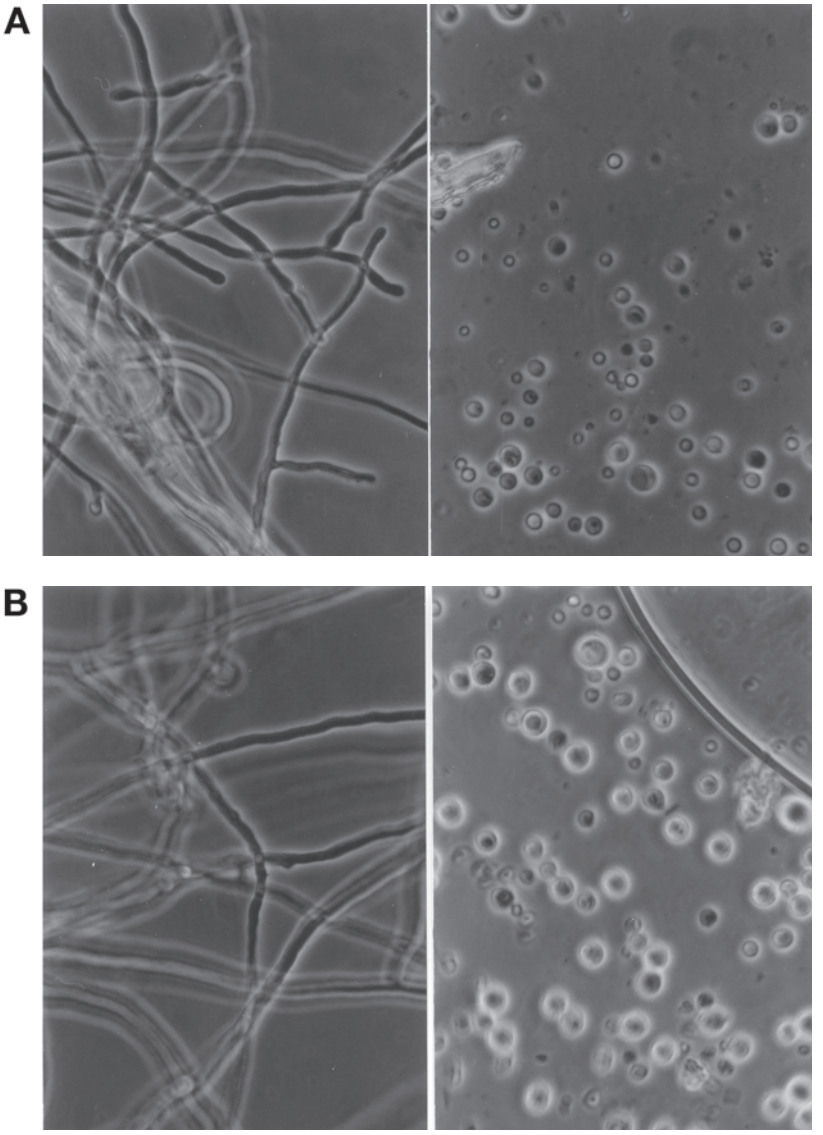

Fig. 1. Photomicrographs of free protoplasts after lytic digestion of mycelia in comparison with normal mycelia of each of the parent strains. **(A)** *T. reesei*, **(B)** *T. harzianum*, **(C)** *T. viride*, and **(D)** *A. niger*.

from one species to another other. The fusion frequency was 1×10^{-3} in the case of fusion between *T. reesei* and *A. niger*. On the other hand, the frequency of intergeneric protoplast fusion between *T. harzianum* and *A. niger* was increased to 2.0×10^{-3}, but it reached the highest frequency when fusion between *T. viride* and *A. niger* was occurred (2.5×10^{-3}).

It was previously mentioned that the formation of protoplasts and regeneration are affected by different factors, e.g., enzymes, time of treatments, mycelial age, regeneration medium, and the like *(5,11,22)*.

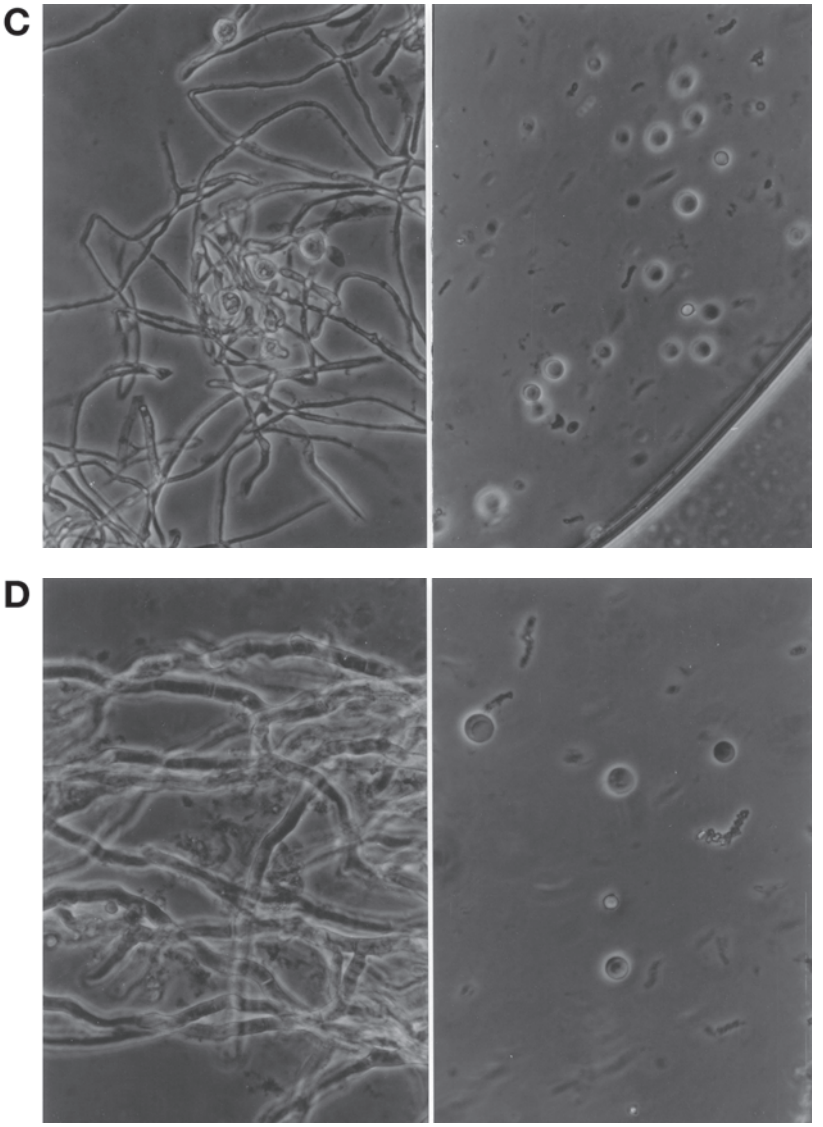

Fig. 1. *(continued)* Photomicrographs of free protoplasts after lytic digestion of mycelia in comparison with normal mycelia of each of the parent strains.

Evaluation of Fusants for Citric Acid and Cellulases Productivity and DNA Contents

Despite the fact that intergeneric protoplast fusion in fungi may yield unstable diploids, some heterokaryons and new recombinants, which are different from the parental cultures, could be obtained. Furthermore, this technique offered the opportunity for overcoming the difficulties of the application of classical hybridization. As was mentioned previously, this

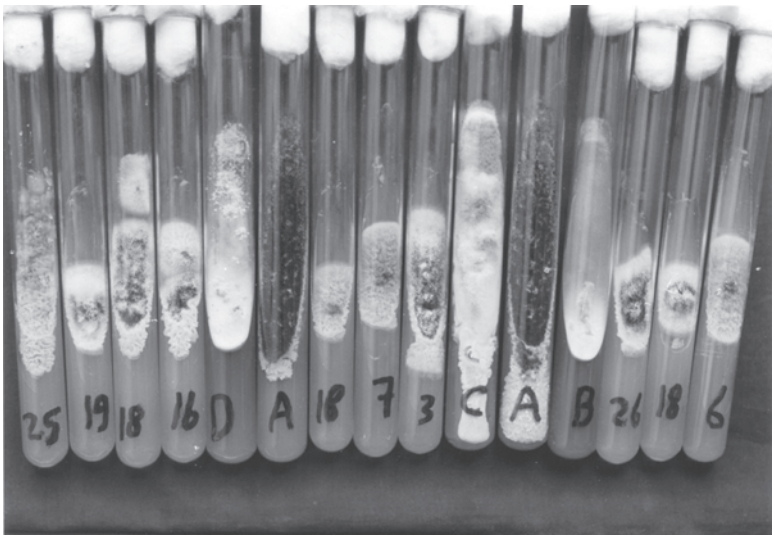

Fig. 2. Some selected fusants, which exhibited morphological variations as a result of three intergeneric protoplast fusion (A: *A. niger*, B: *T. reesei*, C: *T. harzianum*, and D: *T. viride*).

study has explored a new avenue for the direct bioconversion of cellulosic materials as ground rice straw as a new material to citric acid in one stage by the application of intergeneric protoplast fusion.

To achieve this aim, intergeneric protoplast fusion was done in three separated experiments and had succeeded in obtaining two types of intergeneric fusants. Fusant isolates of the first type are haploid and those of the second type showed to be heterokaryons. The stabilities of the fusants obtained were examined by successive subcultures on selective and nonselective media. Figure 2 shows some stable fusants, which differed in morphological forms, from three different intergeneric crosses.

The first fusion was carried out between *T. reesei* NRRL 18670 as the cellulases producer and *A. niger* NRRL 599 as the citric acid producer. Fused cells were spread on selective medium containing 200 ppm of Hg^{++}, 500 ppm of Zn^{++} as metal ions, 0.2% (w/v) 2-deoxyglucose as a catabolite repressor, 100 μg/mL of cycloheximide and 10 μg/mL of 5-fluorouracil as antifungal agents. After the incubation period, only 20 fusants were obtained from this cross and all of them proved to be resistant to the selected markers. Out of these 20 fusants, 11 were recombinants and 9 fusants were heterokaryons, i.e., reverse to their parents and the production was the same as both parents. Table 2 presents the cellulases and citric acid productivities, DNA contents of the parents, and the recombinant fusants. Out of the 11 fusants, two (1/2 and 1/3) were the same as the parent P2 (*A. niger*) in citric acid productivity. On the other hand, the other fusants were found to have more citric acid productivity than the parent strain, *A. niger*. One fusant (1/18) produced a threefold amount of citric acid in

comparison with the P2 strain. Furthermore, it produced 300%, 312.5%, and 200% of FPase, CMCase and β-glucosidase, respectively in comparison with the *A. niger* parent. Fusant 1/18 contained the highest amount of DNA (52.5 μg/mL) in comparison with the obtained fusants and the two parents.

Data in Table 2 also showed that the fusant (1/5) produced 100% more citric acid than *A. niger* NRRL 599 strain. On the other hand, it produced 200, 212.5, and 66.7% of FPase, CMCase and β-glucosidase, respectively, more than its parent; *A. niger*, and contained 45.8 μg/mL DNA. Moreover, two fusant (1/25 and 1/26) proved to produce 80% more citric acid than *A. niger*. Furthermore, two fusants (1/1 and 1/7) yielded 50% more citric acid than the *A. niger* strain. On the other hand, they differed in DNA contents (42.8 and 48.5 μg/mL). These findings may be submitted on the evidence that, citric acid and cellulases production and DNA content were not correlated, however the productivity depends on specific DNA content. Three fusants (1/6, 1/13, and 1/17) produced 20% more citric acid than the *A. niger* strain, while, at the same time, these three fusants differed in their DNA contents and cellulases productivity.

The second intergeneric fusion was carried out between protoplasts of the two cultures, *T. harzianum* NRRL 13879, which showed resistance to 300 ppm Hg^{++}, 500 ppm Co^{++}, 500 ppm Cu^{++}, and 500 ppm Zn^{++} as metal ions, and 10 μg/mL 5-fluorouracil (an antifungal agent) and *A. niger* NRRL 599, which proved to be resistant to 250 μg/mL cycloheximide (an antifungal agent) as shown in Table 1. Only 22 colonies were grown on the selective medium and all fusants were tested for their stability by many transplantation steps on the selective and nonselective medium. Out of the 22 fusants only 12 were heterokaryons, whereas the others were stable and recognized as true recombinant colonies. Results in Table 3 indicated that, two fusants (2/13 and 2/15) showed higher cellulases and citric acid productivity than the other fusants and even their parents. They produced threefold more citric acid from that of the parental strain, *A. niger* NRRL 599, whereas their cellulase activities were 200, 187.5, and 266.7% of FPase, CMCase, and β-glucosidase, respectively from those of the *A. niger* strain. In addition, they differed in DNA contents (47.5 and 52.0 μg/mL) respectively. Meanwhile, three fusants (2/4, 2/17, and 2/22) produced 140% more citric acid than the parental strain, *A. niger*, whereas all of them showed the same cellulases activities. On the other hand, the three fusants 2/3, 2/18, and 2/23 gave an increase of about 100% more in citric acid than *A. niger* NRRL 599. Fusants no. 2/2 and 2/7 showed the reverse direction but they produced the same amount of citric acid as the parental *A. niger* strain. Data in Table 3 also showed that the DNA content differed from one fusant to another, which proved that no correlation appeared between DNA contents and the productivities.

On the other hand, the third intergeneric protoplast fusion was carried out between *T. viride* as a cellulases producer and *A. niger* NRRL 599. Fused cells were spread on selective medium containing 300 ppm Hg^{++} and 500 ppm Co^{++}, Cu^{++}, and Zn^{++} as metal ions, 0.2% 2-deoxyglucose as catabo-

Table 2
Cellulases and Citric Acid Productivities for the Intergeneric Recombinants Induced After the Fusion Between *T. reesei* NRRL 18670 and *A. niger* NRRL 599[a]

		Cellulases productivity									Citric acid production		DNA contents
		FPase			CMCase			β-Glucosidase					
Parents	Fusants	U/mL	% From P1	% From P2	U/mL	% From P1	% From P2	U/mL	% From P1	% From P2	g/L	% From P2	µg/mL
P1: *Trichoderma reesei* NRRL 18670		1.0	100.0	200.0	2.0	100.0	250.0	2.0	100.0	133.3	Not test	–	32.5
P2: *Aspergillus niger* NRRL 599		0.5	50.0	100.0	0.8	40.0	100.0	1.5	75.0	100.0	5.0	100.0	42.8
	1/18	1.5	150.0	300.0	2.5	125.0	312.5	3.0	150.0	200.0	15.0	300.0	52.5
	1/5	1.5	150.0	300.0	2.5	125.0	312.5	2.5	125.0	166.7	10.0	200.0	45.8
	1/25	0.8	80.0	160.0	1.5	75.0	187.5	3.0	150.0	200.0	9.0	180.0	49.0
	1/26	1.0	100.0	200.0	1.5	75.0	187.5	3.0	150.0	200.0	9.0	180.0	49.8
	1/1	1.0	100.0	200.0	1.0	50.0	125.0	2.0	100.0	133.3	7.5	150.0	42.8
	1/7	1.0	100.0	200.0	1.0	50.0	125.0	2.5	125.0	166.7	7.5	150.0	48.5
	1/6	0.5	50.0	100.0	0.8	40.0	100.0	2.5	125.0	166.7	6.0	120.0	35.5
	1/13	0.5	50.0	100.0	1.5	75.0	187.5	2.0	100.0	133.3	6.0	120.0	38.8
	1/17	0.5	50.0	100.0	1.0	50.0	125.0	2.5	125.0	166.7	6.0	120.0	44.5
	1/2	1.0	100.0	200.0	1.0	50.0	125.0	1.5	75.0	100.0	5.0	100.0	35.5
	1/3	1.0	100.0	200.0	1.0	50.0	125.0	1.5	75.0	100.0	5.0	100.0	45.5

[a]Arranged up to down according to citric acid production.

Table 3
Cellulases and Citric Acid Productivities for the Intergeneric Recombinants Induced After the Fusion Between *T. harzianum* NRRL 13879 and *A. niger* NRRL 599[a]

		Cellulases productivity									Citric acid production		DNA contents
		FPase			CMCase			β-Glucosidase					
Parents	Fusants	U/mL	% From P1	% From P2	U/mL	% From P1	% From P2	U/mL	% From P1	% From P2	g/L	% From P2	μg/mL
P1: *Trichoderma herazianum* NRRL 13879		1.0	100.0	200.0	1.0	100.0	125.0	2.5	100.0	166.7	Not test	–	35.5
P2: *Aspergillus niger* NRRL 599		0.5	50.0	100.0	0.8	80.0	100.0	1.5	60.0	100.0	5.0	100.0	42.8
	2/13	1.0	100.0	200.0	1.5	150.0	187.5	4.0	160.0	266.7	15.0	300.0	47.5
	2/15	1.0	100.0	200.0	1.5	150.0	187.5	4.0	160.0	266.7	15.0	300.0	52.0
	2/4	1.0	100.0	200.0	1.5	150.0	187.5	3.5	120.0	200.0	12.0	240.0	49.0
	2/17	1.0	100.0	200.0	1.5	150.0	187.5	3.5	140.0	233.3	12.0	240.0	49.5
	2/22	1.0	100.0	200.0	1.5	150.0	187.5	3.5	140.0	233.3	12.0	240.0	49.5
	2/3	1.0	100.0	200.0	1.5	150.0	187.5	3.0	120.0	200.0	10.0	200.0	42.8
	2/18	1.0	100.0	200.0	1.0	100.0	125.0	3.5	140.0	233.3	10.0	200.0	44.2
	2/23	1.0	100.0	200.0	1.0	100.0	125.0	3.5	140.0	233.3	10.0	200.0	44.2
	2/2	1.0	100.0	200.0	1.0	100.0	125.0	1.5	60.0	100.0	5.0	120.0	38.8
	2/7	0.8	80.0	160.0	1.5	150.0	187.5	1.5	60.0	100.0	5.0	100.0	36.5

[a]Arranged up to down according to citric acid production.

lite repressor, and 1.0 µg/mL miconazole and 10 µg/mL 5-fluorouracil as antifungal agents. After the incubation period, only 24 colonies appeared on the surface of the hypertonic selective medium. They were tested for their stability by many transplantation steps on the selective and nonselective medium. Out of these colonies only 14 fusants were heterokaryons and each reversed to one parent. On the other hand, the other colonies (10 fusants) were true recombinants and characterized as haploids on the basis of their DNA contents. Results in Table 4 proved that, in addition to stability, all obtained fusants showed recombinations concerning the three enzymes responsible for cellulose degradation and citric acid production. Ten fusants showed the ability to produce different amounts of FPase, CMCase, and β-glucosidase and they were divided into four classes concerning citric acid productivity. The first class contained fusant no. 3/7, which produced 140% more citric acid than the parent *A. niger* strain and contained 49.5 µg/mL DNA. The second class gave an increase of about 100% more citric acid than the *A. niger* NRRL 599, containing the three fusants (3/4, 3/5 and 3/6). On the other hand, the third class included four fusants (3/1, 3/18, 3/19, and 3/25), which gave 60% more citric acid than the *A. niger* strain. The fourth class contained two fusants (3/3 and 3/16), which produced the same amount of citric acid as the parent *A. niger* NRRL 599 strain. All of the different fusants differed in DNA contents, which, in time, proved that no correlation appeared between cellulases and citric acid productivities.

During the last decade, it appeared that the sources of fuels are limited and hence, the search for other sources is of great importance. At the same time, the unlimited sources of agricultural wastes also appeared. The conversion of these cellulosic materials to sugars or directly to citric acid is the best solution for using this unlimited source of such fuels and organic acid needed for both medical and industrial products. Results obtained from this study showed that none of the fusants obtained from three intergenric protoplast fusions showed citric acid less than that of the parent *A. niger* NRRL 599. However, three fusants showed citric acid productivity of 200% more than that of the parent *A. niger*. Out of them, one fusant was obtained from the first fusion and the others were obtained from the second fusion.

Halos et al. *(19)* and Pham and Saturnina *(4)* isolated hybrids as Co^R and Hg^R colonies via intergeneric protoplast fusion between *P. funiculosum* and *T. reesei*. They found that the progeny of the two fusants indicated that they have higher CMCase and FPase activities than the parental strains. Furthermore, Kirimura et al. *(9)* found that two types of intergeneric fusants using protoplast fusion between *A. niger* and *T. viride* produce citric acid and cellulases, respectively, were isolated. The first type was haploids and the second was heterokaryons. On the other hand, the intergeneric protoplast fusion technique between *T. reesei* and *S. cerevisiae* to produce ethanol from direct bioconversion of cellulosic materials was also applied by Kumari and Panda *(3)* and Srinivas et al. *(10)*. Whereas, Kvesitadze et al. *(5)* applied the intergeneric protoplast fusion between *Allescheria terrestris* and

Table 4
Cellulases and Citric Acids Productivities for the Intergeneric Recombinants Induced After the Fusion Between *T. viride* and *A. niger* NRRL 599[a]

		Cellulases productivity									Citric acid production		DNA contents
		FPase			CMCase			β-Glucosidase					
Parents	Fusants	U/mL	% From P1	% From P2	U/mL	% From P1	% From P2	U/mL	% From P1	% From P2	g/L	% From P2	µg/mL
P1: *Trichoderma viride*		1.0	100.0	200.0	2.0	100.0	250.0	1.0	100.0	66.7	Not test	–	33.5
P2: *Aspergillus niger* NRRL 599		0.5	50.0	100.0	0.8	40.0	100.0	1.5	150.0	100.0	5.0	100.0	42.8
	3/7	1.0	100.0	200.0	2.0	100.0	250.0	4.0	400.0	266.7	12.0	240.0	49.5
	3/4	1.0	100.0	200.0	2.0	100.0	250.0	3.0	300.0	200.0	10.0	200.0	48.0
	3/5	1.0	100.0	200.0	2.0	100.0	250.0	3.0	300.0	200.0	10.0	200.0	45.5
	3/6	1.0	100.0	200.0	2.0	100.0	250.0	3.0	300.0	200.0	10.0	200.0	46.5
	3/1	0.8	80.0	160.0	2.5	125.0	312.5	2.5	250.0	166.7	8.0	160.0	45.0
	3/18	0.8	80.0	160.0	2.5	125.0	312.5	2.5	250.0	166.7	8.0	160.0	45.0
	3/19	0.8	80.0	160.0	2.5	125.0	312.5	2.5	250.0	166.7	8.0	160.0	44.5
	3/25	1.0	100.0	200.0	2.0	100.0	250.0	3.0	300.0	200.0	8.0	160.0	46.0
	3/3	0.8	80.0	160.0	1.5	75.0	187.5	1.5	150.0	100.0	5.0	100.0	38.5
	3/16	0.8	80.0	160.0	1.0	50.0	125.0	2.0	200.0	133.3	5.0	100.0	43.5

[a]Arranged up to down according to citric acid production.

A. niger to construct a recombinant culture. They found that the morphology of the new culture was similar to that of *A. niger*, whereas its intercellular glucose oxidase activity was twofold higher and could synthesize a thermally stable extracellular endoglucanase similar to that of *A. terrestris*.

Concerning DNA contents, results obtained proved that there were no correlations between DNA and either of citric acid or cellulases production. Furthermore, it was also noticed that DNA of any one of all the isolated fusants differed from that of the two parents. In addition to their genetic stability, all the fusants were shown to be haploids.

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