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ETHANOL PRODUCTION FROM CELLULOSE VIA INTERGENERIC PROTOPLAST FUSION BETWEEN *Trichoderma reesei* AND *Saccharomyces cerevisiae* FUSANTS

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ABSTRACT

The present study aimed to investigate the possibility of inducing new genetic recombinants having the ability to ferment cellulose into ethanol upon fusion of *T. reesei* and *S. cerevisiae* protoplasts. Twelve stable fusants were induced and tested for ethanol as well as cellulase enzymes productivity. Out of these fusants 8 isolates produced ethanol with amounts ranged from 45% (F/4 and F/5) to 90% (F/7) of the parent *S. cerevisiae,* whereas the remaing 4 did not produce ethanol. Meantime these none ethanol producing fusants showed cellulase activities of the three enzymes (FPase, CMCase and β-glucosidase).

On th other hand, 3 fusants (F/1, F/6 and F/7) out of the eight ethanol producer showed cellulase activities of these three enzymes, whereas the other five fusants (F/2, F/4, F/5, F/9 and F/10) showed cellulase activities of the two enzymes FPase and CMCase only. None of these 12 fusants overyieled the parental strain *T. reesei* (S/26) in cellulases production.

Keywords : *Trichoderma reesei, Saccharomyces cerevisiae* Cellulase enzymes, Ethanol, Intergeneric cross, Protoplast fusion.

INTRODUCTION

The industrial fungus *Trichoderma reesei* is well known as an efficient producer of cellulolytic enzymes which hydrolyze cellulose into glucose, meantime, the baker yeast *Saccharomyces cerevisiac* can ferment this sugar into ethanol. The aim of the present work is an attempt to induce and select certain new genetic recombinant fusants which had the capacity to ferment cellulose directly into ethanol, upon the intergeneric protoplast fusion between these two organisms.

Halos *et al.* (1989) and Pham and Halos (1990) used intergeneric protoplast fusion to obtain a hybrid between *Penicillium funiculosum* strain Thom MG-171 and *T. reesei* RUTC30 strain, which produce high levels of β-glucosidase and CMCase, respectively. Cobalt – resistance and mercury – resistance markers were introduced in *P. funiculosum* and *T. reesei;* respectively, through the induction of mutations. Protoplasts of these fungi were fused and screened for hybrids as Co^R and Hg^R colonies. They tested Co^R Hg^R fusants for the production of cellulases and found that activity of various fusants was in general lower than that of their parental strains. However, preliminary results with the second generation or the progeny of the two fusants indicated that they have higher CMCase and FPase activities than the parental strains.

In addition, Kirimura *et al.* (1990) obtained two types of integeneric fusants upon protoplast fusion between *Aspergillus niger* yang no. 2 and y-

(b) (producing citric acid) and *T. viride* M5S51 (producing cellulase). The fist type was haploids prototrophy showing the *A. niger* type morphology, whereas those of the second type were heterokaryons showing mixed morphologies between those of *A. niger* and *T. viride*. Regarding CMCase and β-glucosidase production, 31 fusant strains of the heterokaryon type showed lower enzymes activity than *T. viride*, while 17 fusants showed higher productivities than *T. viride* M5S51.

Kumari and Panda (1994) isolated 201 fusant strains using two different combinations of intergeneric hybrids between *T. reesei* QM9414 and *S. cerevisiae* MCIM 3288 in an attempt to produce ethanol from a cellulosic substrate by a single-stage process. Two fusants out of them (M14 and M62) showed the highest synthesis of ethanol from filter paper cellulose producing 0.0149 and 0.0191 g/L, respectively. These two fusants also had significant extracellular CMCase activity. They found that some fusants were either diploids or heterokaryons. Srinivas *et al.* (1995) studied the optimal conditions to produce ethanol from direct bioconversion of cellulosic materials to ethanol by using intergeneric fusants of *T. reesei* and *S.cerevisiae*. However, the ethanol produced by the fusants was very low compared to the *Saccharomyces*-ethanol process.

Kvesitadze *et al.* (1996) used protoplast fusion between the thermophilic strain *Allescheria terrestris* and the mesophile *A.niger* to construct a recombinant culture characterized by the properties inherited from their parental cultures and also by new properties. The morphology of the new culture was similar to that of *A. niger*, whereas its intracellular glucose oxidase activity was two fold higher. In addition, the new culture could synthesize a thermally stable extracellular endoglucanase similar to that of *A. terrestris*. Vazques *et al.* (1997) succeeded in obtaining yeast-like hybrids between the yeast *Pichia stipitis*, a xylose-fermenting species, and each of *Fusarium moniliforme*, and *T. reesei* by fusion of the yeast protoplasts and the filamentous fungi nuclei. Fusants were selected using the ability to utilize xylan as one selection marker or nitrate nitrogen as the other marker. They suggested that fusion of yeast protoplasts with fungal nuclei is a promising technique for transferring genes from genetically dissimilar organisms to different species of yeast.

MATERIALS AND METHODS

Fungal Strains :

The fungal strains used in this study were ; *Trichoderma reesei* NRRL 12368 induced mutant (S/26), which was induced and selected by Talkhan *et al.* (2002), and *Saccharomyces cerevisiae* NRRL Y-132, which was kindly provided by the Northern Regional Research Laboratory, Dept. of Agric., Illionois, U.S.A.

Media :

The original strain of *T. reesei* and its selected mutant (S/26) were tested for their cellulases activity using four different fermentation media and proved to produce high amounts of the enzyme on the following medium :

- Fermentation medium (FM) : as mentioned by Haapala *et al.* (1995).
- Protoplasting medium (PM): as reported by kumari and Panda (1994). It was used for the induction of protoplasts from *T. reesei* strains.
- The cultivation medium for *S. cerevisiae* protoplasting (CMP) : It contained (g/L) malt extract, 3, glucose, 50; yeast extract, 3 and peptone, 5. The initial pH of the medium was maintained at 6.5 to 6.8 with 1M Na OH solution.
- Hypertonic, selective and regneration medium (HM) : as mentioned by Kumari and Panda (1994) . It contains the same of the (PM) with the addition of cellulose, as a carbon source instead of glucose, 0.7 MKCl and one or more of the antifungal agents used.
- Protoplsts and fusion buffers : according to (Gomori, 1955). Phosphate buffer (25mM, pH 5.8) was used in protoplast preparation, while glycine (0.05M) NaOH buffer (pH 7.5) was used in protoplast fusion.
- Yeast pre-treatment solution :It contains : EDTA-Na_2, 20mM; Tris-HCl, 200 mM; β-mercaptoethanol, 100 mM and pH 7.6.

Yeast protplasting buffer :

It contains : KH_2PO_4, 8mM; sodium citrate, 16 mM; KCl, 600 mM and pH 5.8.

Enzymes :

The Novozyme 234 and snail enzyme were used through this study for protoplasts preparation.

Isolation of antifungal resistant strains :

For the isolation of antifungal resistant strains, protoplast medium (PM) and antifungal agents were used separately, concentrations of the used antifungal agents were added as follows ; Nystatin 10µg/ml; griseofuluin 250 µg/ml; cycloheximide 100µg/ml; benonyl 5 µg/ml and miconazole 5 µg/ml. A part of mycelium or yeast cells from each fungal strain was inoculated on the surface of the antifungal medium plates, the plates were incubated at 28°C for six days. Colonies, which exhibited resistance or sensitivity to a specific antifungal agent were recorded and retested again on the same antifungal dose to be sure of their respose stability.

Protoplast formation of filamentous fungus T. reesei :

Spore suspension from the fungal strain was inoculated with 50 ml of PM in 250 ml flasks and incubated on a rotary shaker (120rpm) for 20 hours at 28°C. After incubation, the mycelium was collected by centrifugation, then washed twice with 0.7M KCl in phosphate buffer, 25 mM, (pH 5.8) and then resuspended in (50 mg/ml) phosphate buffer containing 0.7 M KCl and 8 mg/ml Novozyme 234. The lytic mixtures were incubated at 30°C with gentle shaking for up to two hours. Protoplasts were detected microscopically as osmotically sensitive spherical bodies. The protoplast mixture was centrifuged at 4000 rpm for five min at 4°C, then the supernatant was

discarded and the protoplasts were resuspended immediately in a sterile phosphate buffer containing 0.7M KCl.

Protoplast formation of *S. cerevisiae* :

Yeast cells were grown in 250 ml Erlenmeyer Flask containing 50 ml yeast protoplast medium and incubated on a rotary shaking incubator (120 rpm) at 30°C for 18 hours. Cells were collected by centrifugation and washed twice with sterile distilled water. Washed cells were resuspended in the pre-treatment solution, then the suspension was incubated for 20 min. at 35°C with gentile agitation. After incubation, cells were centrifuged again and resuspended in a protoplasting buffer containing snail enzyme (1% w/v) and incubated under shaking (120 rpm) in a water bath at 35°C. Cells were checked periodically, using microscope for the formation of protoplasts. The conversion of cells into protoplasts was completed within one hour of incubation. Protoplasts were centrifuged at 3000 rpm for 5min and resuspended in protoplasting buffer.

Technique of protoplasts fusion :

Protoplasts of each parental strain, at equal amounts were mixed and centrifuged at 3000 rpm for five min. The residue (mixture of protoplasts) was suspended in 2 ml of a solution 30% (w/v) polyethylene glycol 6000 (PEG) in 0.05 M glycine-NaOH buffer (pH 7.5) containing 0.05 M $CaCl_2$. $2H_2O$. The mixture was allowed to stand at 30°C for 30min. Then the suspension was centrifuged at 3000 rpm for five min., and the residue was washed with phosphate buffer containing 0.7 M KCl and centrifuged at 3000 rpm for five min. The residue was resuspended in 1ml of osmotically balanced phosphate buffer and serially diluted with 0.7 M KCl. Samples, each of 0.5 ml of the dilutions were added to tubes containing $CaCl_2$-agar, mixed immediately and overlayed on the hypertonic, selection and regeneration medium.

Isolation of fusants :

During the present study, PEG treated protoplast suspensions were plated onto an antifungal selective medium, which was supplemented with one of the antifungal agents, griseofulvin, cycloheximide, nystatin, benomyl or miconazole. Plates were incubated at 28°C until colonies were grown on the surface of the plates. They were considered as complementary fusants. They were transplanted and subcultured several times onto selective medium before further studies. All fusants were tested for their stability by many transplantation steps. Stable fusants were then grown on fermentation medium for determination of their enzyme activities.

Determination of ethanol productivity :

Determination of ethanol was carried out by chemical oxidation (Arthur and Wright, 1969). Samples were steam distilled into acidified $K_2Cr_2O_7$ solution. Oxidation of ethanol to acetic acid was completed by heating. Untreated dichromate was determined by titration with ferrous ammonium sulphate solution to almost become clear green. At this point, 3

drops of 1.10 phenanthroline was used as indicator and titration was continued up to the end point (change was from blue-green to brown). Because of ferrous ammonium sulphate solution is oxidized by air, blank determination should be performed daily by using potassium dichromate solution. Ferrous ammonium sulphate solution that has been standing in burette over 30 min. was discarded. Ethanol determination was calculated according to the following equation :

$$\% \text{ Ethanol by volume} = 25.00 - [25.00 - \frac{\text{Sample titer}}{\text{Blank titer}}]$$

Cellulases determination :

Enzyme activities were assayed in the culture supernatant obtained by centrifugation for five min at 5000 rpm under cooling in an eppendorf centrifuge. A half ml of the clear supernatant was diluted in 4.5 ml of 0.05 M citrate buffer, pH 4.8. The dilution was used to assay the enzyme activities.

Enzymatic degradation of filter paper (FPase) :

A piece of filter paper (Whatman No., 1,50 mg) was added to 0.5 ml of 0.5 M citrate buffer, pH 4.8, then 0.5 ml of appropriately diluted enzyme preparation was also added and the mixture was incubated for one hour at 50°C. Since one activity unit (FPU) liberates 1μmol reducing sugar in one minute (Miller, 1959 and Vaheri *et al.,* 1979), then reducing sugars were measured colorimeterically with the dinitrosalisylic acid (DNS) method at wavelength of 570 nm using glucose as a standard (Miller, 1959).

Carboxymethyl cellulose hydrolyzing activity(CMCase) :

A half ml of 1% carboxymethylcellulose in 0.05 M citrate buffer, (pH 4.8,) and 0.5 ml of diluted enzyme preparation were mixed and incubated at 50°C for 30 min. The reaction was stopped with 3 ml DNS reagent for reducing sugars, and the color can be obtained after heating for ten min and measured at wavelength of 570 nm. One unit of enzyme liberates 1μmol reducing sugars in one min (Miller, 1959 and Vaheri *et al.,* 1979).

β-Glucosidase activity :

The determination of β-glucosidase activity was performed with P-nitrophenyl-β-glucoside (Sigma Co.) as substrate, 900 μl of mM P-nitropheny1-β-D-glucoside in 0.05 citrate buffer (pH 4.8) and 100μl diluted enzyme were mixed. After incubation at 50°C for 10min, 1ml of 1M $NaCO_3$ was added. The liberated p-nitrophenol was determined from its absorbance at 400 nm. Enzyme activity unit was defined as the amount of enzyme that liberates 1μmol p- nitrophenol under the assay conditions in one minute (Vaheri *et al.,* 1979).

RESULTS AND DISCUSSION

Collection of genes from different species in one cell by protoplast fusion is an efficient method to improve the productivity of some important industrial microorganisms. The fungus *T. reesei* is a hypercellulases producer, while *S. cerevisiae* ferment sugar into ethanol. The present study

aimed to combine the properties of these two fungi in a new recombinant organism having the ability of producing ethanol from cellulosic farm wastes in one direct process.

To achieve this aim, an intergeneric protoplast fusion was carried out between the highest cellulases producer mutant of *T. reesei* (S/26), which showed resistance to both antifungal agents griseofulvin and benomyl, and *S. cerevisiae* (NRRLY-132) which proved to be resistant to the antifungal agents, cycloheximide, benomyl and miconazole.

Formation , fusion and regeneration of protoplasts :

According to the procedures and conditions described under materials and methods, the mycelium of *T. Reesei* and the cells of *S. cerevisiae* were forced to protoplasting. Figure (1) shows free protoplasts after lytic digestion of these strains (b and d).

Fused protoplasts were spread on the selective medium containing the antifungal agents griseofulvin, cycloheximide and miconazole along with cellulose as the sole carbon source. After the incubation period, only 12 colonies were appeared on the surface of the hypertonic selective medium. They were transplanted onto slants and repeatly subcultured for many generations on the selective medium to be tested for both true recombinations and stability.

These 12 fusants were tested for ethanol production on cellulose mineral medium as well as cellulase enzymes in comparison with the parental strains *T. reesei* (S/26) and *S. cerevisiae* as well as the original culture of *T. reesei*(NRRL–12368) and the results are shown in Table (1).

Results proved that, in addition to stability, all obtained fusants showed recombinations concerning the three enzymes responsible for cellulose degradation. While fusants. Nos. F/2, F/4, F/5, F/9 and F/10 showed the ability to produce different ranges of FPase and CMCase, they were on the other hand, non β-glucosidase producers. Furthermore, when these five fusants activities were compared with the productivity of the original *T. reesei* NRRL 12368, it appeared that they are less FPase and CMCase producers. In addition, fusant No. F/6 showed lower FPase, CMCase and β-glucosidase activities in comparison with the original *T. reesei* strain.

On the other hand, fusant F/8, even it was lower FPase and CMCase producer, it showed about 27% β-glucosidase activity more than the original culture *T. reesei* NRRL 12368. However, fusant No. F/1 showed a reverse view, it proved an increase of about 20% in FPase more than the *T. reesei* NRRL 12368, but it gave only 70% and 21% CMCase and β-glucosidase, respectively, of those in the original *T. reesei* strain. Although fusant F/7 produced 128% more in FPase activity, it showed lower productivity by about 27 and 18% of both CMCase and β-glucosidase

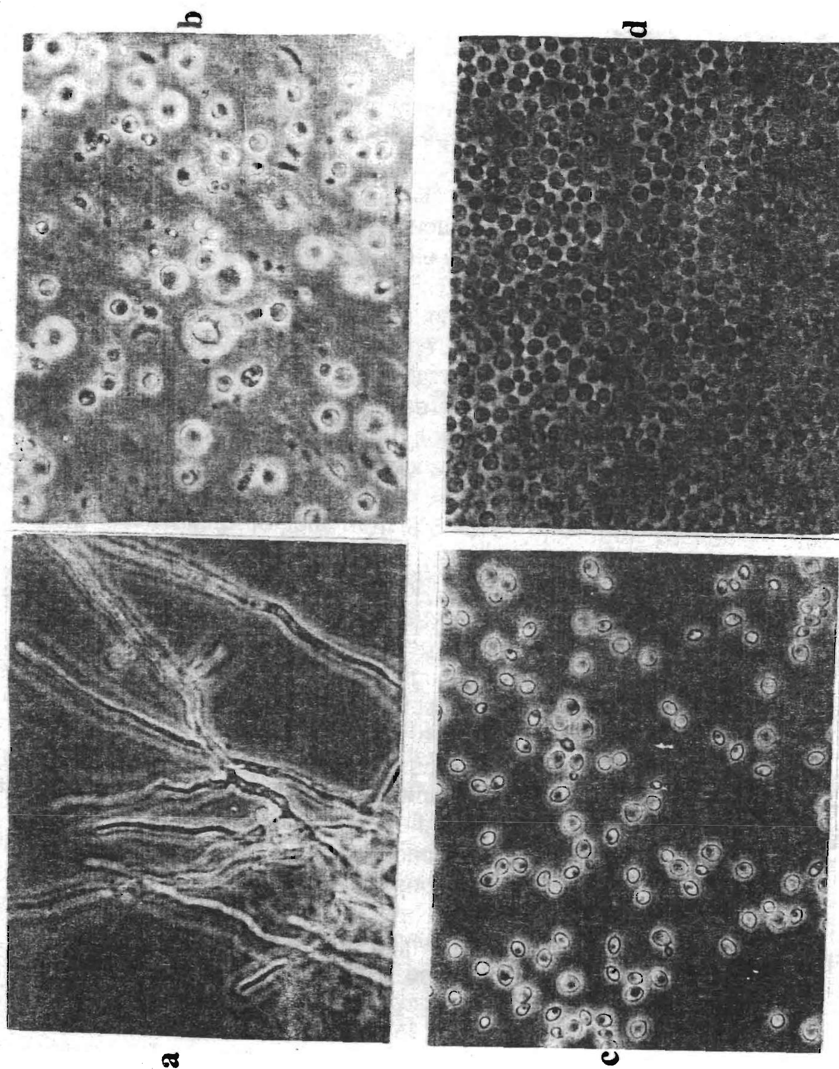

Fig. (1) Protoplasts formation after treatment with lytic enzyme.
a- normal mycelium of *T. reesei.* **b- protoplasts of *T. reesei.***
c- normal cells of *S.cerevisiae.* **d-*S.cerevisiae* protoplasts.**

respectively, in comparison with the original strain *T. reesei* NRRL 12368. In the case of the three fusants Nos. F/3, F/11 and F/12, even they showed resistance on the selective medium, they gave the same enzymes activities of the parental isolate *T. reesei* S/26. Although, none of the obtained intergeneric fusants was non-enzymatic producer like the parent *S. cerevisiae* NRRL Y-132, it appeared at the same time that this intergeneric protoplast fusion did not induce any superior fusant having higher cellulase enzymes productivity than the parental strain *T. reesei* S/26.

On the other side, ethanol productivity for these inergeneric fusants was also determined. Results in Table (1) showed that the three fusants; F/3, F/11 and F/12, which showed the superior activity for the three cellulase enzymes, failed completely to give any ethanol productivity. Moreover, fusant F/8, which showed higher β-glucosidase activity and lower activity of FPase and CMCase, failed also in producing any ethanol amounts. On the other hand, the fusants; F/1, F/6 and F/7 which showed different ranges of cellulase enzymes activities, had the ability to give relatively high ethanol productivity (63.6, 81.8 and 90.9%) respectively, in comparison with the ethanol producer parent *(S. cerevisiae).* Also, fusants; F/2, F/4, F/5, F/9 and F/10, although they showed different ranges of FPase and CMCase activities and were non β-glucosidase producers, they produced intermediate amounts of ethanol ranged from 45.5 to 54.5% from that of *S.cerevisiae* parent. Generally, the low ethanol productivity showed by either *S.cerevisiae* or the obtained fusants may be due to the low level of the carbon source. It also appeared that, none of these intergeneric fusants showed higher ethanol productivity than the parental strain; *S. cerevisiae* NRRL Y-132.

. The obtained results are in agreement with those of Chang *et al.* (1982) who used intergeneric protoplast fusion between *P. chrysogenum* and *C. acremonium* to obtain hybrid strains which probably produce hybrid, solvent-extrastable, β-lactam molecules. They suggested that gene transfer between *Cephalosporium* and *Penicillium* possibly occurred in the prototraphy and altered morphology of these stable fusion products.

On the other hand, Kumari and Panda (1994) isolated 201 fusant strains using two different combinations of hybridization from intergeneric hybrids between *T. reesei* QM91414 and *S. cerevisiae* NCIM3288. They found two fusants (M14 and M62) which showed the highest synthesis of ethanol from filter paper cellulose and that fusants M14 and M56 also had significant extracellular CMCase activity. Also, they found that some fusants were either diploids or heterokaryons. In addition, Srinivas *et al.* (1995) used the intergeneric protoplast fusion technique between *T. reesei* and *S.cerevisiae* to produce ethanol from direct bioconversion of cellulosic materials to ethanol by using intergeneric fusants. While, Kvesitadze *et al.* (1996) applied the intergeneric protoplast fusion between *Allescheria terrestris* and *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 intracellular glucose oxidase activity was two fold higher and could synthesize a thermally stable extracellular endoglucanase similar to that of *A. terrestris.*

Table (1) : Cellulases and ethanol productivity for the intergeneric recombinants induced after the fusion between *T. reesei* S/26 and *S. cerevisiae* NRRL Y-132 , protoplasts.

Parents	Fusants	Ethanol productivity % (V /V)	Cellulases productivity								
			FPase			CMCase			β-glucosidase		
			U/ml	% From higher parent	% From W.T	U/ml	% From higher parent	% From W.T	U/ml	% From higher parent	% From W.T
T.reesei S/26	Original *T.* culture	------	2.5	35.7	100.0	3.7	49.3	100.0	5.7	76.0	100.0
S. cerevisiae NRRL Y-132		------	7.0	100.0	280.0	7.5	100.0	202.7	7.5	100.0	131.6
	*	5.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	F/1	4.5	3.0	42.9	120.0	2.6	34.7	70.3	1.2	16.0	21.1
	F/2	3.0	1.6	22.9	64.0	2.3	30.7	62.2	0.0	0.0	0.0
	F/3	0.0	7.0	100.0	280.0	7.5	100.0	202.7	7.5	100.0	131.6
	F/4	2.5	1.2	17.1	48.0	1.2	16.0	32.4	0.0	0.0	0.0
	F/5	2.5	1.3	18.6	52.0	1.2	16.0	32.4	0.0	0.0	0.0
	F/6	3.5	1.6	22.9	64.0	2.3	30.7	62.2	1.3	16.7	21.9
	F/7	5.0	5.7	81.4	228.0	2.7	36.0	72.9	4.7	62.7	82.5
	F/8	0.0	1.6	22.9	64.0	2.5	33.3	67.6	7.3	96.7	127.2
	F/9	2.7	1.5	21.4	60.0	2.1	28.0	56.8	0.0	0.0	0.0
	F/10	3.0	1.7	24.3	68.0	2.3	30.7	62.2	0.0	0.0	0.0
	F/11	0.0	7.0	100.0	280.0	7.5	100.0	202.7	7.5	100.0	131.6
	F/12	0.0	7.0	100.0	280.0	7.5	100.0	202.7	7.5	100.0	131.6

* = *S. cerevisiae* : production of ethanol on glucose mineral medium.
Fusants : production of ethanol on cellulose mineral medium.

In the case of the intergeneric fusants which showed cellulases activity and failed to give any ethanol productivity, it could be concluded that the gene (s) coding for ethanol system in *S.cerevisiae* could not be introduced into the fusants or could be mutated (altered) during the introduction into these fusants. For the intergeneric fusants which showed ethanol and cellulases activity but were non β-glucosidase producers, the gene(s) coding for β-glucosidase failed to be expressed in these fusants due to mutation or chromosome breakage during the application of protoplast fusion.

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إنتاج كحول الإيثانول من السليلوز بواسطة الدمج البروتوبلاستى بين فطرى التريكوديرما ريساى والسكاروميسز سرفيسيا

فاطمة نبيه طلخان – أحمد محمد البندقلى– على عبده اسماعيل
قسم الوراثة والسيتولوجى – المركز القومى للبحوث – الدقى – القاهرة

يهدف هذا البحث إلى دراسة إمكانية استحداث تراكيب وراثية جديدة قادرة على تحويل السليلوز إلى كحول الإيثانول من خلال تكنيك الدمج البروتوبلاستى بين فطرى التريكوديرما ريساى والسكاروميسز سرفيسيا.

وتشير النتائج المتحصل عليها إلى ما يلى :

- أمكن الحصول على أثنى عشر مندمجة ثابتة تم اختبارها لإنتاج كلاً من كحول الإيثانول وأنزيمات السليوليز وذلك على بيئة تخمر بها السليلوز كمصدر وحيد للكربون.
- أمكن تحديد ثمانية مندمجات لديهم القدرة على إنتاج الإيثانول بكميات تتراوح بين ٤٥% ، ٩٠% مما ينتجه الأب سكاروميسز سرفيسيا فى حين أن المندمجات الأربعة الباقية لم تنتج الإيثانول مطلقاً مشابه بذلك الآب تريكوديرما ريساى.
- أظهرت المندمجات الأربعة ٤ غير المنتجة للإيثانول نشاطاً فى إنتاج أنزيمات السليوليز الثلاثة (β-glucosidase - CMCase - FPase) بينما أظهرت ثلاث مندمجات فقط من الثمانية المنتجة للإيثانول القدرة على إنتاج أنزيمات السليوليز الثلاثة. فى حين أن المندمجات الخمسة الباقية أظهرت القدرة على إنتاج أنزيمى السليوليز (CMCase-FPase) فقط دون الثالث.

ولقد أوضحت النتائج أن جميع المندمجات الأثنى عشر كانت إما مساوية أو أقل من الأب تريكوديرما ريساى فى إنتاج أنزيمات السليوليز ولم تتفوق أى مندمجة على هذا الأب.

وتفيد النتائج المتحصل عليها من هذه الدراسة من الناحية التطبيقية فى إمكان استخدام تلك المندمجات القادرة على تحليل وتحويل السليلوز إلى كحول الإيثانول فى الاستفادة من المخلفات الزراعية السليولوزية مثل قش الأرز وسيقان الذرة وحطب القطن وغيرها. فى إنتاج كحول الإيثانول الذى يستخدم فى صناعات كثيرة بدلاً من حرقها وما ينجم عن ذلك من تلوث للهواء والبيئة بصفة عامة.