FUNGAL GENETICS, CASE OF ASPERGILLUS

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Summary

Classical genetic techniques enabled the selection of an arginine minus auxotroph from each of the mutant strains AW99 and AW96. When crossed, these two deoxyglucose resistant strains yielded a deoxyglucose sensitive diploid. Foreign DNA (via plasmids) can be introduced into the AW99 strain in a stable manner, by electroporation or by polyethylene glycol in the presence of protoplasts. Genes can be directed to a specific site of the genome by Random Enzyme Mediated Integration (REMI).

Pectinases play an important role in industry in the processing of fruit and vegetable juices as they can alter the viscosity and facilitate extraction, filtration and clarification processes [1]. A favourite organism for the production of pectinases is *A. niger* which has been modified by mutation or classical genetics [7] and through molecular genetics [6]. In both cases it was possible to increment production of specific pectinases. Antier *et al.* [3] devised a strategy to increase pectinase production using SSF (coffee pulp) to which the parent strain C28B25 was added. Following UV-induced mutagenesis, mutant strains were derived from *A. niger* C28B25 belonging either to series AW99 [producing maximum pectinase levels in submerged fermentations (SmF)] or series AW96 [producing maximum pectinase level in sold state fermentation (SSF)]. Mutants selected by Antier *et al* were resistant to catabolic repression by saccharose whereas the parent strain was sensitive.. In order to genetically characterise these strains, a master strain was required. Due to incompatibility with known master strains, Loera *et al.*[4] decided to devise their own. It was decided to induce a mutation in the set of DG^R strains in the form of an auxotrophy to arginine. Furthermore, DNA could then be introduced by transformation into the auxotrophic strains of a plasmid pDHG25. This plasmid contained the argB gene which could complement a specific arginine deficient strain. For this study two pectinase hyperproducing strains were selected: AW99 and AW96 (Figure 2). These strains were mutagenized by irradiation with UV light. This step was followed by an enrichment step during which germinating spores on minimal medium were eliminated with the aim of picking up non germinated spores with a higher probability of being auxotrophic [5]. One arginine mutant was obtained from each of the two parent strains, resulting in the following strains : AW99arg $^-$ and AW96arg $^-$. These two strains were then crossed. Loera-Corral [4] obtained a dikaryon of the two DG^R strains (AW99arg $^-$ x AW96arg $^-$.).

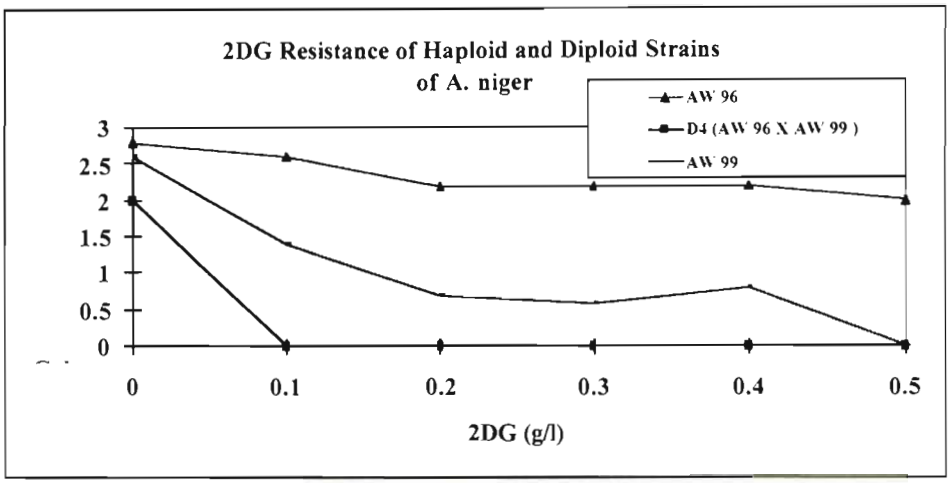


Figure 1. Deoxyglucose resistance of haploid and diploid strains of *A. niger*

The D4 dikaryon shown above in Figure 1 became deoxyglucose sensitive (DG^S); all other phenotypic characteristics were that of strain AW99 [4]. Phenotypes DG^R were complementary to each other (as seen when diploid D4 was obtained) but phenotype AW99 was dominant over AW96. The way in which the phenotype DG^R is associated to the selection of AW96 and AW99 phenotypes is still unknown. Allen *et al.*, [2] have found pleiotropic mutations of *Neurospora crassa* which are associated to DG^R phenotype, mapping as DG^R point mutants in four different loci, and at the same time, show derepressed and modified patterns of invertase and amylase production.

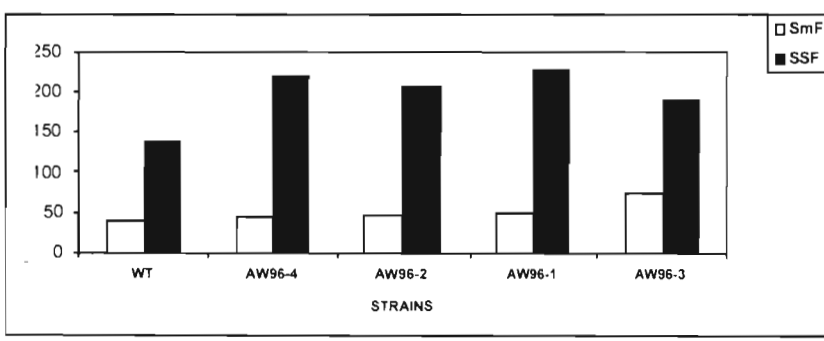


Figure 2. Comparison of pectinase activities of DG^R mutants of *A. niger* C28B25 (WT) isolated at low water activity (a_w = 0.96) and cultured in shake flasks (SmF) and coffee pulp packed bed columns (SSF) according to Antier *et al.* [3]. U PEC are arbitrary enzyme units by viscometry expressed by g of solid substrate (SSF) or dry biomass (SmF).

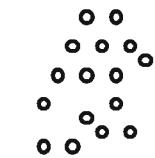
Recent work in our laboratory (Romero *et al.*, 1996 and 1997, unpublished data) has shown that strains AW96 and AW99 are derepressed for the production of pectinase, invertase and amylase, although they were selected on the basis of pectinase over production [3].

Such observations support the existence of general regulatory mechanisms involved in the adaptation of moulds to liquid or solid environments and controlling the yield and quality of enzymes best suited for each kind of culture medium. As a consequence, the selection of strains for the production of enzymes by SSF technique requires the use of specific protocols including the survival to metabolic stress factors, namely, the presence of antimetabolites such as DG or dinitro phenol and also low levels of water activity.

After development through classical genetics of strain AW99argB-, we used molecular genetic methods to test for the introduction of foreign DNA (via plasmids) into AW99arg-. The introduction of foreign genes into fungi has required the setting up of methods adapted from yeasts and bacteria. The arginine auxotroph AW99argB- was used as a host to study the introduction of two plasmids, pDHG25 (an autonomously replicating plasmid; see description in Annex) and pDC1 (an integrative plasmid, see description in Annex). Both plasmids were introduced into the host either by electroporation (see conditions in Annex) or by polyethylene glycol mediated intoduction in protoplasts. A scheme of the latter technique is presented in annex. Additionally, the REMI (random enzyme mediated integration) technique was tested. This technique consists of adding a restriction enzyme (BamH1) along with the DNA to be introduced into the fungal cells. If the fungal genome contains within its DNA, the restiction site, the introduced DNA integrates within that site in the genome. *A. niger* is known to have only one BamH1 site in its genome. Results of these experiments are presented in Annex.

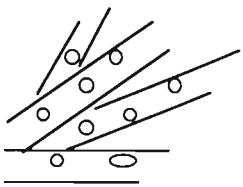
The first conclusion to be made is that it is possible to insert foreign DNA into the AW99arg- strain either by electroporation or by polyethylene glycol (PEG) via protoplasts. In both cases, the number of transformants depends greatly on the type of DNA introduced (linear, circular). The integrative plasmid (pDC1) yields a greater number of transformants, irrespective of the method employed. REMI via electroporation of the integrative plasmid pDC1, seems to be the method of choice. Introduction of the linear pDC1 plasmid in the BamH1 site of the genome induced a phenotypic mutation known as fluffy (data not shown) where no sporulation occurs. The integration of the plasmid therefore disrupted an essential gene involved in conidiation.

Foreign genes can therefore be introduced into the laboratory strains for study. The introduced genes are stable and can either replicate autonomously (if on the pDHG25 plasmid) or integrate into the genome either randomly or specifically (via REMI).



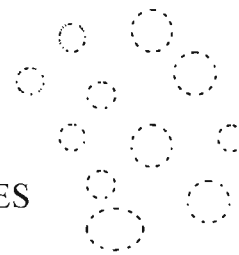
SPORES

14 hr/30°C



YOUNG MYCELIUM (14 hrs)

NOVOZYME 234
(LYTIC ENZYMES *Trichoderma harzianum*)



DNA
PEG

RESULTS FROM ELECTROPORATION

STRAIN: *A. niger dgr*AW99*arg* SPORE CONCENTRATION = 10^7

PLASMID	No. of TRANSFORMANTS /µg DNA	λ PULSE (ms)
Negative control	-	6.7
pDHG25 (circular)	4	5.5
pDHG25 linear/BamHI	32	5.6
pDC1 (circular)	16	6.5
pDC1 linear/BamHI	**40**	**6.3**
pDC1 linear+ 1 U BamHI	**68**	**6.5**
pDC1 linear + 5 U BamHI	12	5.9
pDC1 linear + 10U BamHI	3	6.3

Conditions used for transformation:

Voltage (kv)	Resistance (Ω)	Concentration of DNA (µg)	Recuperation time (hr)
1.0	400	0.25	2.5

RESULTS FROM PROTOPLAST TRANSFORMATION

STRAIN: *A. niger dgr*AW99*arg* SPORE CONCENTRATION = 10^7

TYPE OF DNA	No. TRANSFORMANTS/ µg DNA
Negative control	2-3
Positive control pDHG25 linear BamHI TREATED AT 65°C	**75**
pDHG25 (circular)	24
pDC1 circular	**120**
pDC1 linear /BamHI	18
pDC1 linear /BamHI + 1 unit restriction enzyme	12
pDC1 linear /BamHI + 5 unit	6

restriction enzyme	
pDC1 linear /BamHI + 10 units restriction enzyme	2

STABILITY RESULTS

Strain: *A. niger dgr*AW99*arg*

transformation method: Electroporation

Type of plasmid	Percent stability
pDHG25 circular	60 %
pDHG25 linearized	68 %
pDC1 circular	75 %
pDC1 linearized	70 %
pDC1 linearized + 1 U BamHI	71 %
pDC1 linearized + 5 U BamHI	74 %
pDC1 linearized + 10 U BamHI	67 %

Strain: *A. niger dgr*AW99*arg*

Transformation method: Protoplasts

Type of plasmid	Percent stability
pDHG25 circular	70 %
pDHG25 linearized	65 %
pDC1 circular	79 %
pDC1 linearized	83 %
pDC1 linearized + 1 U BamHI	83 %
pDC1 linearized + 5 U BamHI	81 %
pDC1 linearized + 10 U BamHI	79%

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