MOLECULAR TECHNIQUES APPLIED TO FUNGAL STRAIN UPGRADATION CAPABILITY RELATED TO SSF CULTURES

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Summary

Mutant strains derived from *A. niger* C28B25 and belonging to series AW99 [producing maximum pectinase level in submerged fermentation (SmF)] and series AW96 [producing maximum pectinase level in solid state fermentation (SSF)]were compared concerning pectinase properties and thermal stability. Pectinases produced by AW96 exhibited increased thermal stability compared to AW99. Results support the idea that not only the quantity but also the quality of the enzymes can be modified when SSF or SmF techniques are used. The use of differential display polymerase chain reaction may enable the isolation of solid state fermentation-specific genes.

Our group is trying to use molecular methods in order to study a fundamental problem of fungal fermentation, that is, the way fungi adapt to grow and reproduce in liquid or solid media. With ever growing new uses for enzyme production in SSF, there is a need to improve the basic understanding of the physiology of molds used to produce enzymes by SSF. This way, over fifteen years of SSF studies can help to give a qualitative jump on the physiology and biochemistry of filamentous fungi with specific reference to their interaction with a specific solid substrate. In fact, filamentous fungi in general (whether perfect or imperfect, but growing as filamentous structures or mycelia) have a remarkable adaptability to the specific type of substrate. Such kind of adaptations are most surely controlled by the transcription and expression of different sets of genes. Identification of the « trigger » genes controlling those fungal adaptations will help to use modern techniques of genetic engineering to clone, transfer and express new genes from any biological source coding for enzymes and other useful products to be produced by different fermentation techniques.

Previous work in our laboratory [1] with a wild-type strain of *A.niger* called C28B25 led to the isolation of two different kinds of mutants resistant to 2-deoxyglucose (2-DG), namely the DG^R AW96 class of strains and DG^R AW99 class of strains. Interestingly, these mutant strains behave in an opposite manner to each other that is, DG^R AW96 proved to be endopectinase hyperproducers when cultivated by SSF but not as much when cultivated by SmF whereas DG^R AW99 hyperproduced endopectinase when cultivated by SmF but were poor pectinase producers when cultivated by SSF [3]. Thus it can be considered that DG^R AW96 strains are « **adapted** » to produce pectinase on SSF and DG^R AW99 are « **adapted** » to SmF production.

Alazard and Raimbault [6] found that amylase activity produced by *A. niger* using SSF technique was more heat tolerant than the one produced by SmF technique. Acuña-Argüelles

and coworkers [2] confirmed such observation by measuring pectinase activities produced by SSF and SmF and using an *A. niger* CH4 strain.

In an effort to approach at a molecular level, the nature of the differences observed, zymographic patterns of pectin hydrolases and pectin esterases produced by a wild strain *A. niger* C28B25 and mutants AW96 and AW99 were obtained.

For the detection of *in situ* pectinolytic activity, and to study the effect of heat, extracts from the growth of each strain in either SSF or SmF were obtained and divided into two samples. Only one sample was heated at 90° C for 60 s. The samples were electrophoresed and pectinolytic activity was detected *in situ*. AW99 lacked a pectin esterase band at 70 Kd when compared to the other strains. Neither the AW96 nor the wild-type strains hydrolytic activities could be detected before heating and only the AW99 mutant exhibited a slight hydrolytic activity band. Nevertheless, heating of the SSF extracts resulted in an apparent **activation** of an additional hydrolytic activity.

Only two low molecular weight pectin esterase activities could be detected in SSF extracts but none showed the 70kDa activity that had previously been observed in SmF extracts. All esterase activities were lost by heating the extracts.

The SSF culture technique, thus produced pectin hydrolases that had the remarkable property of needing a brief thermal treatment in order to show catalytic activity and were different from the ones produced by the SmF culture technique which led to thermal-sensitive pectin hydrolases. Apparently, thermal sensitivity of this latter activity was greater than for those of the wild type or AW96 strains which provides additional evidence for the presence of discreet differences at the molecular level. The exact nature of the differences, whether resulting from modifications of the polypeptide chain or differences in glycosylation patterns, has yet to be elucidated.

Furthermore, thermal stability of *in vitro* enzyme activity was studied by viscometry [mainly endopolygalacturonase (endoPG) activity]. EndoPG activities from AW96 strain produced by either SmF or SSF techniques declined slowly with thermal treatment whether in the presence or absence of substrate (Figure 1.)

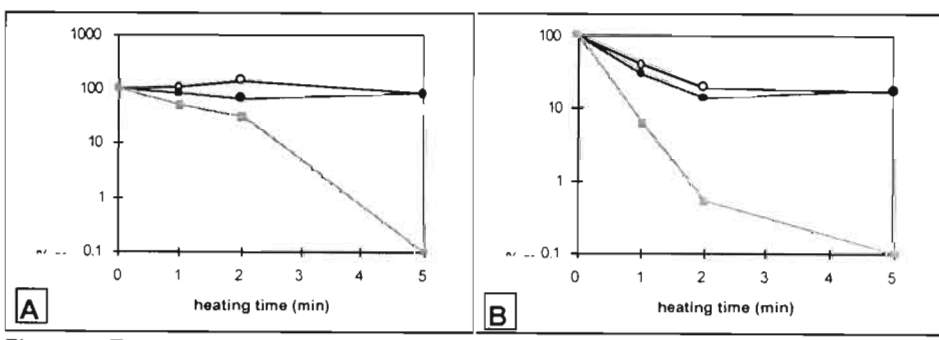

Figure 1. Thermal stability of *in vitro* endopectinase activity of SmF extracts (A) and SSF extracts (B) in the presence (closed symbols) or absence (open symbols) of pectin (5g/L). AW96=square symbols, AW99=round symbols. T=92°C. Note the logarithmic scale on the axis.

Activity from AW99 produced by SmF showed a trend without any significant difference from that of AW96 extracts when heated in buffer. In contrast, the same extract

exhibited a much more pronounced inactivation slope when heated in the presence of substrate (Figure 1A). Samples from SSF exhibited a more pronounced inactivation slope especially in the case of AW99 (Figure 1B). In this case, no significant differences were found in the presence or absence of substrate. In all cases, endoPG activity produced by AW96 strain was more thermostable than that produced by AW99 strain.

Exopectinase activities (Figure 2) produced by both types of mutants in SmF had similar trends when heated in the presence or absence of substrate (Figure 2A).

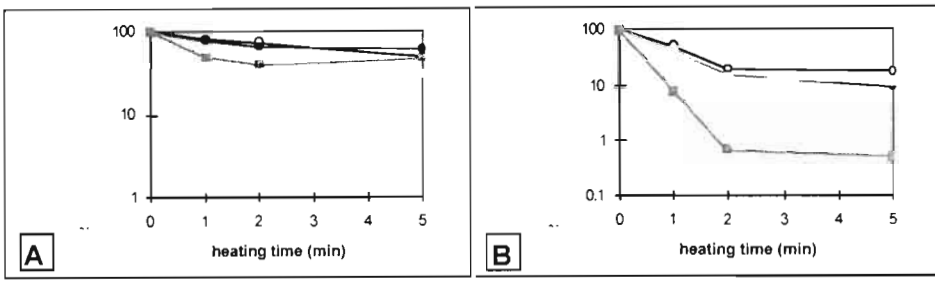

Figure 2. Thermal stability of *in vitro* exopectinase activity of SmF extracts (A) and SSF extracts (B). in the presence (closed symbols) or absence (open symbols) of pectin (5g/L). AW96=square symbols, AW99=round symbols. T=92°C. Note the logarithmic scale on the axis.

Likewise, exopectinase activities had similar trends in extracts obtained from both strains by SSF when heated in buffer (Figure 2B) however, extracts from the AW96 strain showed and increased thermal sensitivity when heated in the presence of substrate. Results therefore indicated that pectinolytic enzymatic complexes produced by each type of mutant strain were different when produced by SmF or SSF.

Pectin hydrolases produced by SSF technique were more resistant to heat denaturation than those produced by SmF technique. Pectin esterases were, instead heat labile in a similar way when produced either by SSF and SmF techniques. Pectinase activity produced by AW96 mutants was more heat tolerant than that produced by AW99 [2].

Thermal tolerence of pectin hydrolases is an interesting property when analyzed by elecrophoretic zymography. Zymographic differences were related to the use of SSF or SmF techniques and the nature of each given strain. For example, the SSF technique produced a pectin hydrolase band requiring previous heating in order to have activity in the gel. The nature of the heat tolerence of the protein produced by SSF requires further basic work. Perhaps this could be related to the activation of a zymogen or the inactivation of a thermolabile inhibitor associated with the native protein.

In relation to pectin esterase activites, SmF produced a distinct band at 70 kDa which was absent in cultures obtained by SSF. These results give further support to the idea that each given fermentation technique is responsible for the production of different pectinase patterns [2, 7]. On the other hand, pectinase activities measured in crude extracts by viscometry showed very important differences between culture techniques and strains. For example, pectinase activites produced by the AW96 strain were more thermostable than those

produced by the AW99 strain. This could be related to earlier reports which suggested that enzymes produced by SSF are more thermostable than those produced by SmF [2,6,7].

The effect of heating on pectinase activities measured by viscometry did not seem to be the same as that revealed by the zymograms, but this may be related to the interaction in the former among several enzymes and soluble materials present in the reaction mixture; nevertheless, thermal stability analysis of enzyme extracts helps to distinguish among different enzymatic phenotypes produced by different strains and culture techniques.

The molecular basis of those differences would require purification and detailed biochemical characterization of each given enzyme but opens some interesting questions on the way the molds adapt to solid and liquid culture techniques using perhaps a different set of genes or modifying their expression in a differential way.

In brief, although both types of mutant strains (AW96 and AW99) were selected for their resistance to 2-deoxyglucose (DG^R), the results showed that both classes are in fact different and also that the DG^R phenotype may not be directly involved with the complex patterns of physiological derepression and enzyme production which is in agreement with the hypothesis of having pleiotropic mutations associated with the DG^R phenotype.

As stressed in the introductory comments, identification of the « trigger » genes controlling fungal adaptations to SSF would greatly help in the understanding of the mechanisms that control the adaptability of molds. We intend to test in the near future, a relatively new technique known as differential display polymerase chain reaction (DD-PCR) in order to determine whether it is possible to identify genes that are specific for solid state fermentation. The technique has been successfully used in our laboratory to identify genes that regulate caffeine degradation in *A. niger*. Differential display is a powerful screening technique used for the detection and identification of differentially expressed genes [4] related to solving developmental, environmental [5] and hormonal problems. In this method, two or more RNA's (from SSF and SmF cultures) are used as templates to generate cDNA. Subsequently the cDNA fragments are amplified by PCR using an arbitrary primer in the presence of a radiolabeled nucleotide (dNTP). After separation by denaturing polyacrylamide gel electrophoresis the gels are fixed and dried. Differentially amplified cDNA's are identified by autoradiography. To produce sufficient DNA for further analysis, the differentially expressed DNA is eluted from the gel and reamplified and then cloned. Northern analysis then confirms that a given clone is SSF-specific.

The above mentioned results seem to support the idea that not only the quantity but the quality of enzymes can be modified when SSF or SmF techniques are used. This may be accomplished either by turning on and off different sets of genes coding for different polypeptides, by controlling the edition of the same polypeptides (i.e., differences in glycolsylation) or by a combination of both kinds of mechanisms. Distinction between such hypothese the use of techniques such as DD-PCR which is part of the present research program in our laboratory.

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