Biological Resource Centres and the Use of Microbes

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Correlation between a deep clearing test and quantitative methods for lipolytic activity of filamentous fungi

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Summary

The deep clearing test (DC) is a simple method for measuring the relative lipolytic activity of fungi. The depth of the clear zone below the fungal colony provides a visual measure of the lipolytic activity on a continuous cumulative basis. The depth of clearing was determined for four commercial enzyme concentrations and compared by correlation analysis with results obtained by a titrimetric method and a colorimetric method for measuring lipolytic activity. Good correlation coefficients were obtained between DC and either the titrimetric (R^2 = 0.9373) or the colorimetric method (R^2 = 0.9579). Using these data a putative lipolytic activity was determined for seven strains of filamentous fungi from Micoteca da Universidade do Minho (MUM) culture collection.

Keywords: Lipolytic activity; Deep clearing test; Fungi

Introduction

Lipolytic enzymes are widely found among animals, plants and microorganisms. Lipases (EC 3.1.1.3) are acylglycerol hydrolases and catalyse the degradation of fats and oils through the hydrolysis of the ester bond between glycerol and the acyl moiety. These are versatile enzymes with both physiological significance and large industrial potential. Besides the hydrolysis of fats and oils, their applications include the modification of fats, flavour enhancement in food processing, resolution of racemic mixtures, specialty organic synthesis, and chemical analysis. Due to their increasing impact in bioprocessing

there is a demand for enzymes with an optimal range of catalytic specificities and required properties for the various applications (Sharma *et al.*, 2001). There are various methods for detecting lipases. Rapid microbial screening methods include the detection of lipolytic activity in agar plates. It has been shown that the diameter of halos formed due to the reaction products can be linearly correlated with the logarithm of the enzyme concentration, therefore allowing quantitation of lipase activities (Kouker and Jaeger, 1987). The deep clearing test (Lima *et al.*, 1991) is a simple and convenient method for measuring the relative lipolytic activity of fungi. This method is currently used in the Micoteca da Universidade do Minho culture collection (MUM). It consists of measuring the depth of the clear zone below the fungal colony in opaque columns of agarized tributyrin, providing a visual measure of the relative lipolytic activities of the screened fungi. In this study the deep clearing test was compared with quantitative titrimetric and colorimetric analysis.

Materials and Methods

Microorganisms and culture conditions

The fungi screened belong to Micoteca da Universidade do Minho culture collection. *Aspergillus niger* MUM 92.13 and MUM 01.01, *Aspergillus versicolor* MUM 01.03, *Penicillium crustosum* MUM 01.04, *Phanerochaete chrysosporium* MUM 95.01, *Trichoderma viride* MUM 97.54 and *Trichoderma* sp. MUM 00.22 were revived from silica gel storage and grown on malt extract agar (Blakeslee). The cultures were then transferred to Czapek Dox agar (Saccharose 0.3 %, $NaNO_3$ 0.3 %, K_2HPO_4 0.1 %, $MgSO_4.7H_2O$ 0.05 %, KCl 0.05 %, $FeSO_4.7H_2O$ 0.001 %, $ZnSO_4.7H_2O$ 0.001 %, $CuSO_4.5H_2O$ 0.0005 %, agar 2 %) and incubated at 30 °C to produce the inocula for the enzyme assays.

Preparation of enzyme solution

Fungal lipase Type VII from *Candida rugosa* (Sigma, L-1754) with an activity of 875 U.mg^{-1} solid was dissolved in Tris-HCl 50 mM, pH 7.2 at 5 mg.ml^{-1}. Insoluble material was removed by centrifugation. The stock solution was kept in aliquots at $-$ 20 °C.

Enzyme assays

Deep clearing test

Agar (oxoid no. 1) 1 % (w/v) or Czapek Dox mineral medium with 1 % agar, were autoclaved at 121 °C for 15 min. After cooling to 60 °C filter sterilised tributyrin was added in the concentration of 0.1 % (w/v). The mixture was emulsified with a Heidolph Diax 900 blender and dispensed into 16 x 160 mm test tubes in the upright position. The tubes were rapidly chilled in a mixture of water and ice (Lima et al., 1991). Ten replicate tubes were inoculated with 100 µl of 10, 20 50 and 100 µg commercial lipase solution. For the screening of the fungal strains three replicate tubes were inoculated with a 10 mm disk cut from the edge of a 7 day old colony with a sterile cork borer. The test tubes were incubated at 30 °C and the depth of clearing (mm) was measured every 24 h up to 5 days incubation.

Microplate p-NPB assay

Activity was determined using p-nitrophenyl butyrate (Sigma N-9876) as substrate. The reaction mixture contained 200 µl 50 mM Tris-HCl buffer pH 7.2, 25 µl substrate in 2-propanol (0.56 mM) and 25 µl enzyme solution [modified from Castillo et al. (1999)]. Enzyme activity was measured continuously for 3 min at 30 °C by monitoring the release of p-nitrophenol at 405 nm using a SLT Spectra III reader. One unit of enzyme activity was defined as the amount of enzyme that releases 1 micromole of p-nitrophenol per hour. Each data point is the average of 8 determinations.

Titrimetric assay

The reaction mixture contained 10 ml assay buffer (2 mM Tris-HCl pH 7.2, 0.05 M NaCl, 0.05 M $CaCl_2$) mixed with 10 ml substrate emulsion. The emulsion was prepared by homogenising 5 ml tributyrin, 5 ml gum arabic 5 % (w/v) and 90 ml distilled water with a Heidolph Diax 900 Blender, 4× 30 s with 30 s intervals [adapted from Jacobsen et al. (1989)]. The reaction was started by addition of 20-200 µl enzyme solution and measured at 30 °C and pH 7.2 by titration with 0.01 M NaOH using a microtitrator from Metrohm. One unit of enzyme activity was defined as the amount of enzyme that releases 1 micromole of free fatty acid per hour. The assays were performed in duplicate.

Results and Discussion

The deep clearing test provides a visual measure of the lipolytic activity on a continuous cumulative basis. Depth of clearing has linear time dependence. The method is further validated by the existing linear relationship between the depth of clearing (mm) and lipase amount (μg), the parallel lines indicating a constant rate of lipolysis (Fig. 1).
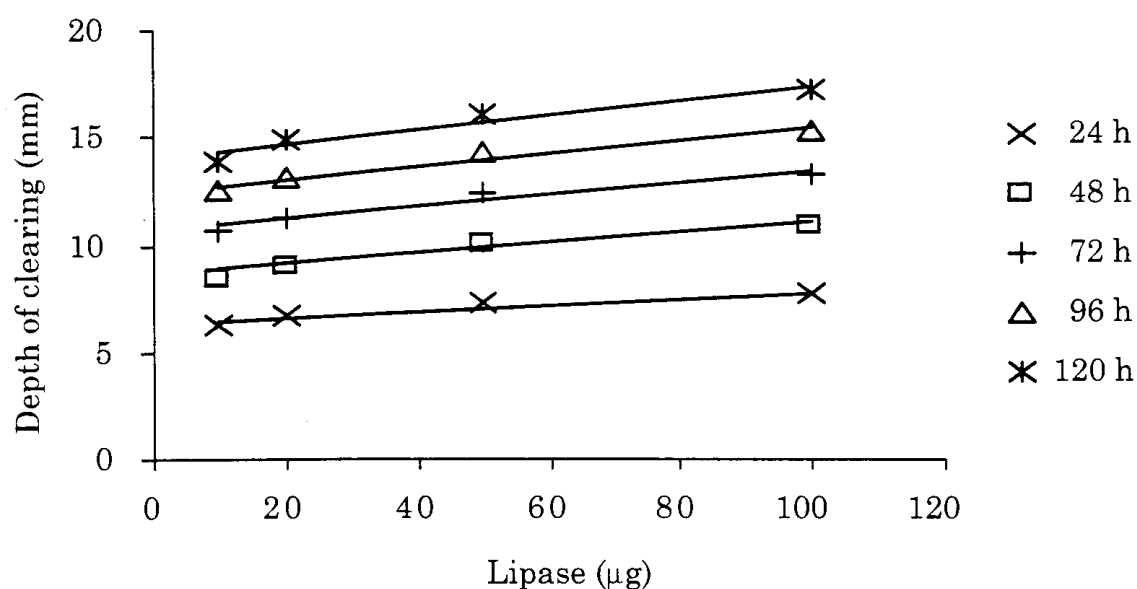

Figure 1. Behaviour of the depth of clearing with different lipase solutions.

Although tributyrin is not considered a true lipase substrate it is convenient for the present purpose. This substrate is easier to handle and the products of its degradation are soluble allowing the formation of a sharp, well defined interface between the clear and opaque zones. For this reason and to better establish a correlation between methods, titrimetric analysis was performed also using tributyrin as lipolytic substrate while colorimetric analysis used p-nitrophenyl butyrate.

The correlation coefficients obtained from data comparison are respectively 0.9373 for deep clearing vs. titrimetric analysis (Fig. 2-A) and 0.9579 for deep clearing vs. colorimetric analysis, for a range of 10-100 μg lipase (Fig. 2-B).
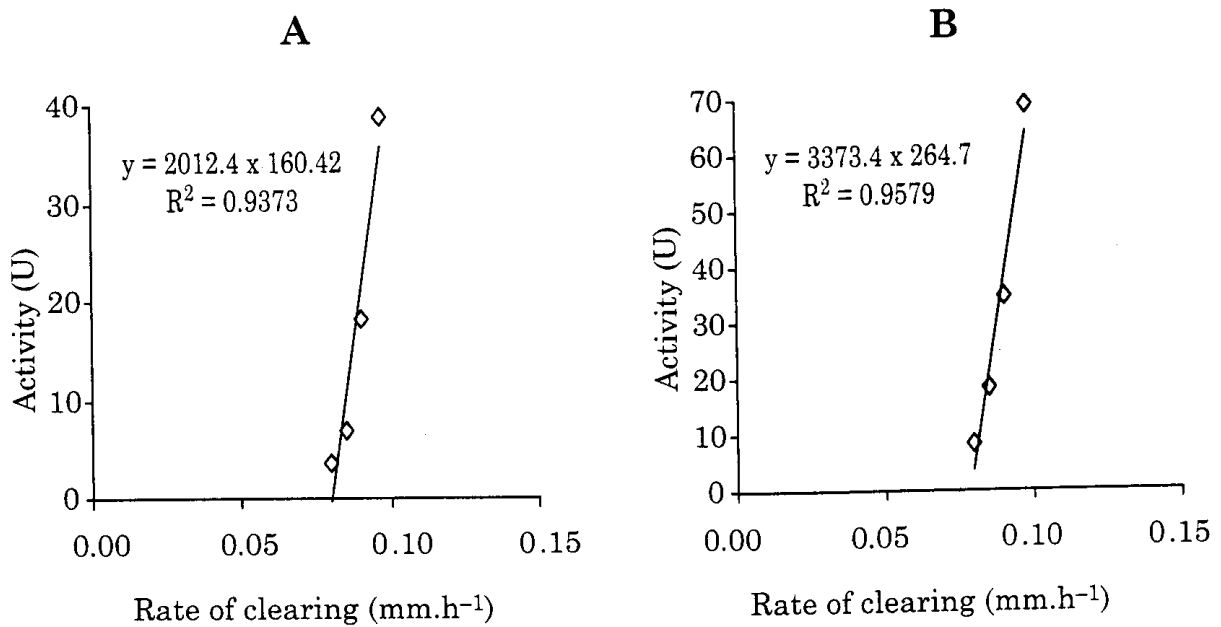
Figure 2. Correlation of results of A: titrimetric analysis and deep clearing test; B: colorimetric analysis and deep clearing test.

Using the relationships illustrated in Figure 2 it is possible to estimate a putative lipolytic activity for the strains tested (Table 1).

Table 1. Depth of clearing (DC) at 120 h incubation and putative lipolytic activity (U) for the seven screened strains

Strain number	DC (mm)	Titrimetry (U)	Colorimetry (U)
97.56	6.0	< 0.6	< 5
92.13	6.3	< 0.6	< 5
01.01	8.7	< 0.6	< 5
00.22	12.0	14.6	28.7
01.03	12.0	17.6	33.8
01.04	11.3	28.4	51.9
95.01	10.3	28.6	52.1

Strains 95.01 and 01.04 exhibit the higher putative lipolytic activities, when strains 00.22 and 01.03 were the ones with higher depth of clearing at 120 h. We believe this is due to the fact that the fungi were

not pre-adapted to the lipolytic substrate and therefore will have a lag period that may be different for each strain. As a result, it is possible that a strain which started enzyme production earlier and consequently exhibits higher initial and final values of depth of clearing, can present a smaller rate of hydrolysis.

Conclusions

A primary screening method should be sensitive, simple to perform, inexpensive, capable of assessing a large range of samples, and predictive but not too restrictive. We believe the deep clearing test to fulfil these criteria being a reliable method for the screening of lipolytic fungi. The correlation found between this method and quantitative titrimetric and colorimetric analysis makes this test more robust, allowing the conversion of measured rates of clearing into lipolytic activity values. Further studies are ongoing towards the establishment of the deep clearing test as a possible quantitative test.

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