Isolation of *Aureobasidium pullulans* from Zimbabwean sources and glucosidase activities of selected isolates

RN Okagbue*, E Mwenje, T Kudanga, M Siwela and T Sibanda

Department of Applied Biology and Biochemistry, National University of Science and Technology, PO Box AC 939, Ascot, Bulawayo, Zimbabwe

* Corresponding author, e-mail: bio11@telconet.co.zw

Received 1 September 1999, accepted in revised form 26 April 2000

The yeast-like fungus *Aureobasidium pullulans* (DeBary) Arnaud has been isolated and identified for the first time in Zimbabwe from diverse sources including fruits, leaves and commercial manure. Selective enrichment of an appropriate substrate using a minimal salts broth with shaking for two days at 25°C followed by plating on a corresponding solid medium, proved to be a reliable method for isolating the fungus. Extracellular α and β-glucosidases produced in broth cultures by selected isolates were generally low; yields ranged from 0.7–1.4 units/ml and 1.7–2.5 units/ml respectively. Of six (mainly complex) nitrogen sources, NaNO₃ supported the highest level of extracellular α-glucosidase in *A. pullulans* Mn²⁺ despite a very low final pH (2.42). However, addition of Tween 80 to a 24 hour old culture led to a three-fold increase in yield of extracellular α-glucosidase (approx. 6.0U/ml).

Introduction

*Aureobasidium pullulans* (DeBary) Arnaud is a yeast-like fungus which is usually associated with plant tissue and plays a role in carbon mineralisation processes (Hudson 1972, Burns 1982). Its cultural and microscopic features include variable levels of dark pigmentation on agar media and the presence of septate hyphae, chlamydomospores and budding cells. Since the early seventies, there has been a growing research interest in *A. pullulans* due to its products and properties that have proven or potential economic importance. These include an extracellular polysaccharide pullulan, many hydrolytic enzymes, single cell proteins, tolerance to certain pollution levels and roles in biodeterioration of materials. An extensive literature review is available on the various economic aspects of the organism (Deshpande *et al.* 1992).

The above mentioned literature review covered the global distribution of *A. pullulans* and recognised its widespread occurrence. However, it was stated that the organism seemed to be most common in temperate zones with Britain and the United States being the sources of most reported isolations. A list of countries (covering temperate, mediterranean, arid and tropical zones) which reported the presence of *A. pullulans* was presented. Egypt and South Africa were the only African countries mentioned. We are aware of some enzymatic studies carried out in South Africa on *A. pullulans* (for example, Myburgh *et al.* 1991a and b) but the strain was obtained from an American Culture Collection. Although the presence of *A. pullulans* is at a very low frequency, only about 3% of a total of 27 random samples of Nigerian cassava flour from one locality and none of a total 88 samples from seven other localities yielded the organism (Abba Kareem and Okagbue 1991), isolation of the organism from other sources has not been reported in the African hinterland.

Although strains of *A. pullulans* could differ in certain physiological properties, they have shown consistency in ability to grow on some carbohydrates (Dennis and Buhagiar 1973) and in production of certain extracellular hydrolytic enzymes (Federci 1982). Their production of amylases, xylanases, α- and β-glucosidases has attracted much attention because the enzymes are potentially useful for converting plant polymeric biomass (especially starches and lignocellulosic materials) into single cell protein and biofuels. Therefore, relevant studies on the organism have been intensified in developed countries such as Italy and the United States (see, for example, Federici and D’elía 1983, Saha *et al.* 1993, Saha *et al.* 1994). However, as most third world countries have agro-based economies which produce large quantities of plant biomass and as increased research on biomass conversion strategies have been recommended for them (Anon 1979), it seemed appropriate that *A. pullulans* should be of research interest in third world countries of Sub-Saharan Africa. The aim of this study was to isolate *A. pullulans* from Zimbabwean habitats and to explore the physiological diversity of the isolates including their ability to produce α- and β-glucosidases.

**Materials and Methods**

**Enrichment isolation of *A. pullulans***

Vegetable materials, especially leaves, were collected from certain plants/trees (Table 1) at the main campus of the
Bulawayo Polytechnic and subjected to an enrichment procedure described by Pollock et al. (1992). It involved incubation of the samples in minimal salts broth of the following composition (per litre of distilled water): 1g of (NH4)2HPO4; 0.5g of NaCl; 0.05g of MgSO4.7H2O; 2g of KH2PO4; 0.01g each of FeSO4·7H2O, MnSO4, and ZnSO4·HCl to pH 7.0; and 10g sucrose in 250ml conical flasks and shaken in an orbital shaker at 25°C for two days. The enrichment promoted development of yeast-like cells which remained mainly in suspension when the shaking was stopped. Subsequently, samples of the suspension were inoculated onto agar plates containing the same minimal salts medium and incubated at the same temperature. Colonies which developed after 3–4 days were visually and microscopically assessed for typical *A. pullulans* characteristics such as presence of septate mycelia, blastospores and chlamydospores; development or otherwise, of black/brown pigmentation as the colonies became older, were also noted (De Hoog 1998). Representative colonies with ‘desired’ characteristics were isolated by conventional methods and kept on potato dextrose agar (PDA) slants in the refrigerator.

**Assimilation of carbon compounds by selected isolates**

Twenty of our isolates were tested for the ability to assimilate carbon compounds by using the auxanographic method normally applied to yeasts (Barnett et al. 1983). The basal medium used was yeast nitrogen base (Difco) and incubations were at 25°C for 7 days. In our experience assimilation patterns of *A. pullulans* on carbon sources can be reliably assessed with auxonograms in a manner similar to that of well known yeasts which can be concluded in 7 days (Barnet et al. 1983).

**Cultivation of selected *A. pullulans* isolates for production of extracellular glucosidases**

Seven isolates, arbitrarily chosen, were cultivated in shake flasks. For α-glucosidase production, 50ml of medium (in duplicate 250ml Erlenmeyer flasks) of the following composition (g/l): maltose, 10; NaN3, 2.0; FeSO4·7H2O, 0.01; MgSO4·7H2O, 0.5; NaCl, 0.5; yeast extract (Oxoid), 0.4, was inoculated with a loopful of each organism taken from the slant culture and incubated at 25°C on a rotary shaker at 200rpm for two days. Thereafter, 2ml of the resulting inoculum culture was transferred into 100ml of medium of the same composition contained in 500ml flasks which were again incubated with shaking under the same conditions. After four days, the yeast cells were removed by centrifugation at 1 800g for twenty minutes. Biomass was washed with distilled water; dried at 105°C for 4h and then weighed in dry pre-weighed aluminium pans. The supernatant was used as the crude enzyme preparation and was preserved with 0.2g/l sodium azide if it was not immediately used and stored at 4°C. The amount of biomass per ml of culture was determined and that formed the basis for computing the amount of enzyme produced per gram biomass produced in the culture. For β-glucosidase production, the procedure was similar to that of α-glucosidase production except that the medium contained in addition, KH2PO4, 1g/l and bactocellobiose, 1g/l was substituted for maltose.

**Enzyme assays**

For α- and β-glucosidase activities, respectively, the reaction mixtures contained 1ml of 1% maltose or 0.1% bactocellobiose, 1ml of 50mM acetate buffer pH 4.5 or pH 5.0 and 1ml of crude enzyme preparation. Incubations were at 50°C for 30min. For both assays, the amount of glucose liberated during the incubation was oxidised using dinitrosalicylic acid (DNSA) reagent. One millilitre of DNSA reagent and 0.3ml of Rochelle salt (sodium-potassium tartrate) were added and the mixture was boiled for 5min. After cooling to room temperature, the amount of reducing sugar was measured by spectrophotometric absorbance readings at 575nm and a glucose standard curve was used. One unit of enzyme was defined as the amount of enzyme that liberated 0.01mg of reducing sugar as glucose per minute under these conditions. Enzyme unit per gram biomass was computed based on the biomass per ml which was determined as has been explained earlier.

**Effect of nitrogen sources on production of extracellular α-glucosidase by *A. pullulans* Mn2**

An inoculum culture was prepared with *A. pullulans* Mn2 as described above for β-glucosidase production except that the medium contained FeSO4·7H2O at a concentration of 0.001g/l. Additionally, KH2PO4 at 1.0g/l was included and pH was adjusted to 5.0 using 1M HCl. Two millilitres of the inoculum culture was used to inoculate each of the duplicate flasks containing 100ml of a basal fermentation medium (of the same composition as the inoculum culture medium) supplemented with one of the following nitrogen sources: NaN3, 2.0g/l; (NH4)2SO4, 2.0g/l and peptone, tryptone, yeast extract and meat extract (Lab Lemco), each 5.0g/l. The fermentation flasks were incubated on the rotary shaker at 200rpm at 25°C for four days after which α-glucosidase activities and final pH of the broths were determined.

**Effect of Tween 80 on production of extracellular α-glucosidases by *A. pullulans* Mn2**

The procedure was the same as that in which the effect of
nitrogen sources was examined, except that Tween 80, a surfactant, was added (at 0.2% and 0.4% (v/v) levels) to 24-hour old duplicate fermentation cultures containing NaNO₃ as the sole nitrogen source. A control, in duplicate, was also prepared to measure enzyme production in the absence of surfactants.

Results and Discussion

Our preliminary observations of A. pullulans in this laboratory were purely by chance. Initially the organism was found among yeasts growing on potato dextrose agar (PDA) plates inoculated with samples of juice from locally grown grapes. Subsequently, the organism occurred in platings (on PDA) of ripe fruits of marula plant (Sclerocarya caffra). Again, we obtained some A. pullulans isolates when samples of commercial manure as well as rotten avocado fruits (Persea americana) were plated out to recover probable deteriorative fungi. These chance isolations and absence of information on the occurrence of A. pullulans in this part of Africa (based on Deslipande et al. 1992) prompted us to undertake the screening exercise reported in this work to obtain some reasonably reliable information on its occurrence and on the possibility of its isolation under defined conditions.

Interestingly, virtually all the sources screened by enrichment yielded A. pullulans. The colonies resulting from the enrichment (including those obtained by chance as pointed out above) developed black to brown pigmentation in aged cultures and microscopy revealed septate hyphae, blastospores and chlamydoospores; these attributes are consistent with the description of A. pullulans given by De Hoog (1998). Further, to confirm the identity of the organisms we took cognisance of the characters which have been found useful for distinguishing A. pullulans from A. prunorum and Trichosporon pullulans (Dennis and Buhagiar 1973). Thus our isolates utilised glycerol (unlike T. pullulans) and galactose (unlike A. prunorum).

Other substrates utilised for growth by our A. pullulans isolates were cellobiose, xylose, maltose, sucrose, pectin, xylan, dextrin, arbutin, glucuronic acid and sodium acetate. While the responses of our isolates on most of these carbohydrates are in line with published observations (Dennis and Buhagiar 1973), utilisation of the last three substrates by A. pullulans does not seem to have been published. Unfortunately our isolates failed to assimilate salicin, inulin and lactose, and exhibited variable responses on arbutin and tributylin (Table 2). They also failed to utilise casein, gelatin, vanillin and tributylin; these results are at variance with those reported for the strains examined by Federici et al. (1982). It is possible that the use of auxanograms and seven incubation period in this study had an adverse effect on the abilities of strains to assimilate the complex substrates (casein, gelatin, etc) which were readily utilised by aerobic shaken cultures.

As mentioned earlier, research on production of hydrolytic enzymes including α- and β-glucosidase, by A. pullulans, is being intensified; the later enzymes mediate terminal hydrolytic activities involved in saccharification of starch and cellulose, respectively, for various commercial applications. Table 3 shows that all our selected isolates produced appreciable levels of the two enzymes. However, each organism appeared to produce a significantly higher level of β- than of α-glucosidase under the conditions employed in this study. Unfortunately, the levels of both enzymes produced by all the strains are generally low, the approximate maximum level of β-glucosidase being only 2.5U/ml of broth; this result is in agreement with the observations made on one strain of A. pullulans by Saha et al. (1993, 1994). It should be noted that the cited observations were made in what appeared to be pioneer studies on glucosidase activities of A. pullulans and that the workers also examined the effect of carbon sources on the production of the enzymes by the organism. To supplement their observations we have examined the effects of nitrogen sources and of a surfactant, Tween 80, on yields of α-glucosidase.

Table 4 shows that although all the tested nitrogen sources supported appreciable levels of extracellular α-glucosidase production, the highest yield of the enzyme occurred in presence of NaNO₃ despite a significantly low final pH. This finding contradicts a study (Takii et al. 1995) in which two complex nitrogen sources (meat extract and pep-

| Substrate     | Positive strains | Negative strains | % positive* |
|---------------|------------------|------------------|-------------|
| Glucose       | 20               | 0                | 100         |
| Cellobiose    | 20               | 0                | 100         |
| Xylose        | 20               | 0                | 100         |
| Maltose       | 20               | 0                | 100         |
| Methyl-α-D-glucoside | 0        | 20               | 87          |
| Arbutin       | 20               | 0                | NA          |
| Sucrose       | 20               | 0                | 100         |
| Lactose       | 0                | 20               | 95          |
| Pectin        | 20               | 0                | 100         |
| Xylan         | 20               | 0                | NA          |
| Dextrin       | 20               | 0                | NA          |
| Salicin       | 0                | 20               | 100         |
| Fructose      | 20               | 0                | NA          |
| Galactose     | 20               | 0                | 100         |
| D-mannose     | 20               | 0                | NA          |
| D-ribose      | 20               | 0                | 100         |
| D-glucuronic acid | 20         | 0                | NA          |
| L-arabinose   | 20               | 0                | 100         |
| L-rhamnose    | 20               | 0                | 100         |
| Sodium acetate| 20               | 0                | NA          |
| Xyitol        | 20               | 0                | NA          |
| Mannitol      | 20               | 0                | 100         |
| Ribitol       | 13               | 7                | 100         |
| Inulin        | 0                | 20               | 100         |
| Glycerol      | 20               | 0                | 100         |
| Arbutin       | 13               | 7                | NA          |
| Casein        | 0                | 20               | NA          |
| Gelatin       | 0                | 20               | 100         |
| Tannic acid   | 0                | 20               | NA          |
| Tributylin    | 0                | 20               | NA          |
| Vanillin      | 0                | 20               | 47**        |

NA = Not assayed. *117 isolates were tested, **utilisation of vanillin by strains of A. pullulans was reported by Guiraud et al. (1992).
and fJ-glucosidases and has shown that use of surfactants enhanced release of enzymes into the culture medium. This is consistent with the findings of Reese (1972) that surfactants caused several fungi to produce extracellular, up to 20 fold increases of certain hydrolytic enzymes including cellulase, β-glucosidase and amylase. It was suggested that surfactants enhanced release of enzymes into the culture medium by increasing cell membrane permeability.

Overall, this study has shown that A. pullulans occurs to an appreciable extent on Zimbabwean vegetation (Table 1) and that it can be reliably isolated by enrichment; the observations extend existing knowledge (reviewed by Deshpande et al. 1992) of the global distribution and micro-ecological habitats of the fungus. Also the study has confirmed that A. pullulans strains generally produce appreciable extracellular levels of α- and β-glucosidases and has shown that use of NaNO3 as the nitrogen source in culture medium and addition of Tween 80 (up to 0.4%) to 24 hour-old culture of A. pullulans may boost α-glucosidase content of the broth.

### Table 3: Levels of α- and β-glucosidases produced by local isolates of A. pullulans

| Isolate | α-glucosidase | β-glucosidase |
|---------|---------------|---------------|
|         | units/ml      | units/g biomass | units/ml | units/g biomass |
| Mn-2*   | 1.40          | 500.00        | 2.40     | 857.14          |
| Mn-5    | 0.68          | 226.67        | 2.22     | 740.00          |
| Mn-6    | 0.96          | 342.86        | 2.48     | 885.71          |
| Av-1    | 0.68          | 261.54        | 1.67     | 642.31          |
| Mr-8    | 0.65          | 232.14        | 2.18     | 778.57          |
| Mn-11   | 1.01          | 388.46        | 1.85     | 711.54          |
| Mr-12   | 0.78          | 300.00        | 1.95     | 750.00          |

All results are means of duplicate determinations.

*Codes applied to the isolates are related to their sources, viz. Mn (manure), Av (avocado), Mr (marula leaves); numbers denote isolate number.

### Table 4: Extracellular α-glucosidases production on different nitrogen sources by A. pullulans isolate (Mn*)

| Nitrogen source | α-glucosidase | β-glucosidase | Final pH |
|-----------------|---------------|---------------|----------|
| Tryptone        | 1.73          | 617.86        | 8.20     |
| Peptone          | 1.32          | 471.43        | 6.78     |
| Yeast extract   | 1.53          | 546.43        | 6.08     |
| Meat extract    | 1.50          | 535.71        | 7.32     |
| NaNO3           | 2.08          | 742.86        | 2.42     |
| (NH4)2SO4       | 1.44          | 514.29        | 7.29     |

All results are means of duplicate determinations.

*The initial pH in all cases was 5.00.

### Table 5: Effect of surfactant (Tween 80) addition to the culture medium on extracellular α-glucosidase production by A. pullulans isolate (Mn*)

| Amount of surfactant added | Enzyme yield | R*  |
|---------------------------|--------------|-----|
|                           | units/ml     | units/g biomass |
| None                      | 2.0          | 740.29 | 1   |
| 0.2% Tween 80             | 3.7          | 132.43 | 2   |
| 0.4% Tween 80             | 5.7          | 203.71 | 3   |

### References

Abba Kareem VN, Okagbue RN (1991) Studies on the microbiology of cassava flour. Nigerian Food Journal 9: 85-91

Anon (1979) Microbial Processes: Promising technologies for developing countries. National Academy of Sciences, Washington DC

Barnett JA, Payne RN, Yarrow D (1983) Yeasts: characterisation and identification. Cambridge University Press, Cambridge

Burns RG (1982) Carbon mineralisation by mixed cultures. In: Bull AT, Slater JH (eds) Microbial Interactions and Communities, vol. 1. Academic Press, London, pp 475-543

De Hoog GS (1998) A key to anamorph genera of yeast-like Arch and Euroascomycetes. In: Kurzmann CP, Fell JW (eds) The Yeasts, a Taxonomic Study. Elsevier, Amsterdam, pp 123-127

Dennis C, Buhtagiar RWM (1973) Comparative study of Aureobasidium pullulans, A. prunorum sp. nov. and Thichosporon pullulans. Transactions of The British Mycological Society 60: 567-575

Deshpande MS, Rale VB, Lynch JM (1992) Aureobasidium pullulans in applied microbiology: A status report. Enzyme and Microbial Technology 14: 514-527

Guirand P, Steinman R, Seigle-Murandi F, Benoiguyod J-L (1992) Growth and amylotic activity of Aureobasidium pullulans, A. prunorum sp. nov. and Thichosporon pullulans. Antarctic Microbiology 26; 267-273

Hudson HJ (1972) Fungal saprophytism. The Institute of Biology studies No. 32. Edward Arnold (Publishers) Limited, London

Joyal MJ, Prior BA (1998) A key to anamorph genera of yeast-like Arch and Euroascomycetes. Cambridge University Press. Cambridge

Pollock JT, Thorne L, Armentron RW (1992) Amylolytic enzymes produced by a color variant strain of Aureobasidium pullulans. Applied and Environmental Microbiology 58: 877-883

Saha BC, Freer SN, Bothast RJ (1994) Production, purification and properties of a thermostable β-glucosidase from a color variant strain of Aureobasidium pullulans. Applied and Environmental Microbiology 60: 3774-3780

Saha BC, Silman RW, Bothast RJ (1993) Amylolytic enzymes produced by a color variant strain of Aureobasidium pullulans. Current Microbiology 26: 267-273

Edited by J van Staden