#O-3

Effects of Medium Composition on Morphology and Organic Acid Production in *Phanerochaete chrysosporium*

M.T. Kingsley, R.A. Romine, L.L. Lasure
Pacific Northwest National Laboratory, Richland, WA.

Mark T. Kingsley
Environmental Characterization and Risk Assessment Group
Pacific Northwest National Laboratory
POB 999, MSIN K2-21
Richland, WA 99352
mark.kingsley@pnl.gov
509-375-3965

ABSTRACT

Phanerochaete chrysosporium (Pc) are members of the white-rot group of Basidiomycete fungi. There has been widespread industrial interest in these fungi due to their extracellular production of lignin degrading enzymes (lignin peroxidase (LiP) and manganese peroxidase (MnP)). The Pc bioprocess literature is replete with reports of problems caused by the filamentous nature of the organism; it is sticky and binds to a variety of stirred-tank-reactor (STR) vessel components. This has led to various immobilization and suspension schemes or the use of air-lift fermenters in conjunction with immoblization of the fungus. We have evaluated processes for employing Pc in STRs for the production of organic acids. We are designing processes employing nutritional control to cause Pc to maintain a pelleted morphology. Pc strains ATCC 24725, 32629, 34540, and 34541 were grown in 250 ml baffled flasks (40 mls media, 39C, 250 RPM) or 1.5 or 2.5 L STRs in conjunction with NBS BioFlo 3000 controllers (1 L and 2 L culture volumes, respectively). Both defined (modified Bonnarme and Jeffries) and complex media (Vogel's-YM) were evaluated. Nutritionally controlled morphology was easiest to implement in defined media. High glucose (20%) led to reduced biomass accumulation (vs. 1-2% glucose) and the production of non-sticky mycelial pellets. Media with high glucose caused Pc to grow as non-sticky pellets in flasks and STRs. Organic acids such as succinic, oxalic, lactic and malonic were produced temporally by Pc cultures.

OVERVIEW

• In filamentous fungi, the occurrence and size of mycelial pellets is important in a number of industrially important processes (1-3).

• Major disadvantages of dispersed mycelial growth for commercial production using fungi include increased wall growth and reduced mixing and O_2 exchange. These problems can be rectified, to some degree by growing the mycelial organisms in submerged culture as pellets.

• Growth as pellets is advantageous by lowering medium viscosity and better mass exchange of oxygen and nutrients due to the decreased surface to volume ratio.

• Size of pellets is critical for the formation of secondary metabolites (see 3)

• A significant body of literature exists describing the various extracellular peroxidases produced by the white rot basidiomycete fungus *Phanerochaete chrysosporium* (Pc). These enzymes -- lignin peroxidase (LiP) and manganese peroxidase (MnP) -- were found to be broad spectrum and in addition to potential industrial applications in the paper and forest products industries, were also very useful in the bioremediation of recalcitrant hydrocarbon compounds.

• Dicarboxylic organic acids such as oxalic and malonic acids associated with production of MnP.

• LiP and MnP are produced when the fungus is undergoing secondary metabolism and under nitrogen starvation.

• The goal of these studies was to determine if nutritional control of mycelial morphology could be employed with a conventional Stirred Tank Reactor (STR). Due to the sticky nature of PC mycelial filaments in convential fermentations most work with PC, for enzyme production (LiP and MnP), have employed immobilized fermentation systems (see 4).

METHODS

• Shake flask experiments were conducted in 250 ml baffle flasks agitated at 250 RPM at 39°C.

• Spore inoculum was prepared from 7-8 day old petri plates of PDA-grown ATCC 24725 (grown at 39°C) harvested with 0.85% sterile saline with the aid of a glass hockey stick. Spore counts were determined by hemocytometer. Flasks and fermenters were inoculated at the rate of ca. 1 X 10^5 spores/ml.

• 40 ml of a modified Bonnarme and Jeffries medium (BJA) (1) (Na-acetate substituted for the Na-tartrate buffer which interfered with our ion chromatography analysis for organic acids in the spent media, see Table 1 for details).

• Fermentations were performed in an NBS BioFlow 3000 equipped with a 2.5L vessel, 2L BJA were employed.

• Biomass determined as filtered dry wt., glucose was determined by the Amplex Red Glucose assay (Molecular Probes, Inc.) organic acids were determined by ion chromatography.

TABLE 1. BJA MEDIUM
(modified from (1))

Basal media (contents per liter)	
Glucose	10g (1%), [for 20% add 200g/l, etc.]
KH_2PO_4	2g
$MgSO_4 \cdot 7H_2O$	0.5g
$CaCl_2 \cdot 2H_2O$	0.1g
Tween 20	0.5g
Thiamine HCl	1 mg
Trace element solution (no Mn++) - 70ml/L medium	
Veratryl alcohol	2.5mM (0.42 g/l)
sodium acetate (pH4.5)	20 mM, (1.7 g)
diammonium citrate 0.244g	
(add 20 PPM Mn++), add 1ml of Mn stock to 1 l	
Trace element solution (per liter) [add 70ml/L to medium, above]	
Nitriloacetoc acid	1.5g
$MgSO_4 \cdot 7H_2O$	3g
NaCl	1g
$FeSO_4 \cdot 7H_2O$	0.1g
$CoSO_4$	0.1g
$CaCl_2 \cdot 2H_2O$	0.1g
$ZnSO_4 \cdot 7H_2O$	0.1g
$CuSO_4 \cdot 5H_2O$	0.01g
$AlK(SO_4)_2 \cdot 12\ H_2O$	0.01g
H_3BO_3	0.01g
$Na_2MoO_4 \cdot 2\ H_2O$	0.01g

RESULTS & DISCUSSION

• 20% glucose caused ATCC 24725 to remain as small 0.25 mm (or less) pellets. Unlike growth in media containing 1 or 2% glucose where large pellets and sticky mycelial masses resulted (not shown) the effect of the high glucose was to maintain a tight compact non-sticky pellet which did not adhere either to the sides of baffle flasks nor to surfaces in the bioreactors (Fig 1 a-c).

• High glucose concentrations led to production of filaments with multiple refractile spore-like bodies (Fig 1 b,d,e). Pellets from cultures grown in 1-2% glucose lacked these structures (Fig 1c). A rich medium (Vogels+YM with 20% glucose) led to very short, thinkened filaments which ultimately proved to be sticky and was not used in further studies (Fig 1f).

• Biomass data derived from flask experiments indicated that high glucose was inhibitory to growth reducing overall biomass by 50% relative to low glucose controls. Whether this effect was strictly due to osmolarity is unknown at this time. When grown in the NBS fermenter a fine structured pellet morphology was also maintained.

• Organic acid production is correlated with MnP production, and LiP and MnP production are correlated with secondary metabolism and nitrogen starvation (1,4,5). The desired organic acid for this study (malonate) had a temporal appearance, and production of malonate was inconsistent, while oxalic acid was always observed.

• In the fermenter runs malonate (if produced) accumulated late in the fermentation cycle (see Fig 2a, b). In a number of runs there was good production of other organic acids (such as lactic, succinic or oxalic) there was no malonic detected (see Fig 2c and d). These results are not due to the lowered glucose as malonate was seen in flask experiments at equivalent glucose concentrations.

• In the fermenter, as the glucose concentration of the medium was decreased the tendency for mycelial aggregation and agglomeration on vessel surfaces increased.

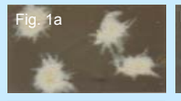


Fig. 1a

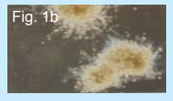


Fig. 1b


Fig. 1c

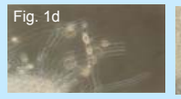


Fig. 1d

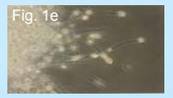
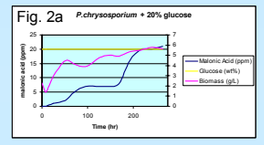
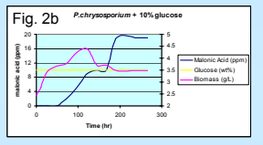
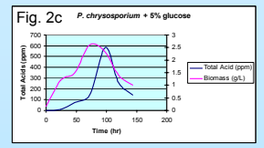


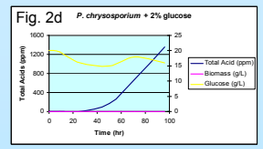
Fig. 1e


Fig. 1f









CONCLUSIONS

• While high glucose concentrations (> 10%) were useful in maintaining a fungal morphology that was amenable to bioprocessing, the high glucose concentrations were inhibitory to the growth of ATCC 24725, lowering overall biomass.

• Glucose assays, in media containing high levels of glucose, showed minimal to no glucose utilization. The quantities of biomass obtained corresponded more closely to the amounts of carbon available (per liter) from the acetate and diammonium citrate supplied as buffer and N-sources, respectively.

• Although high glucose concentrations yielded a desired pellet morphology, ATCC 24725 did not use the glucose supplied in the BJA medium to support biomass growth. Additionally, production of the desired organic acid (malonate) was also variable despite MnP and other secondary metabolite production.

• Malonate was only observed in the fermenter under the high glucose conditions, however, the current yields are impractical for bioprocess when considering the quantities of glucose supplied (and not utilized),

• In the bioreactor at lowered glucose concentrations led to increased filamentous growth and to a lack of malonate being produced in the suite of organic acids.

• Since MnP and LiP production (and consequently the organic acids accompanying MnP production) are strongly linked with the C/N ratio of the media and especially nitrogen starvation, media affecting the carbon balance may be assumed to affect secondary metabolism. From that standpoint other mechanisms to control the fungal morphology in the fermenter, especially in this particular instance might yield better results.

• However, high glucose did force the fungus to maintain small, tight pellets, which from a bioprocess standpoint, in a stirred tank reactor, are highly desirable.

Literature Cited

1. Bonnarme, P., and T. W. Jeffries. 1990. Mn(II) regulation of lignin peroxidases and manganese-dependent peroxidases from lignin-degrading white rot fungi. Appl. Environ. Microbiol. **56:**210-217.
2. Braun, S., and S. E. Vecht-Lifshitz. 1991. Mycelial morphology and metabolite production. TIBTECH **9:**63-68.
3. Hulst, A. C., M. M. Meyer, H. Breteker, and J. Tramper. 1989. Effects of aggregate size in cell cultures of *Tagetes patula* on tiophene production and cell growth. Appl Microbiol Biotechnol **30:**18-25.
4. Jimenez-Tobon, G. A., M. J. Pennickx, and R. Lejune. 1997. The relationship between pellet size and production of Mn(II) perxoidase by *Phanerochaete chrysosporium* in submerged culture. Enzyme Microb Technol **21:**537-542.
5. Linko, S. 1992. Production of *Phanerochaete chrysosporium* lignin peroxidase. Biotechnol. Adv. **10:**191-236.

Pacific Northwest National Laboratory
Operated by Battelle for the U.S. Department of Energy

Battelle
. . . Putting Technology To Work