Biotechnology Letters Vol 10 No 7, 497-502 (1988)
Received May 25

IMPROVEMENT OF GROWTH OF RHIZOPUS OLIGOSPORUS ON A MODEL SOLID SUBSTRATE

D.A. Mitchell*[1], H.W. Doelle[1] and P.F. Greenfield[2]
[1]Department of Microbiology and [2]Department of Chemical Engineering,
University of Queensland, St. Lucia, Queensland, 4067, Australia.

SUMMARY

Metal ions and cassava extracts stimulated growth of Rhizopus oligosporus on a model solid substrate. A large improvement in protein content was obtained by simultaneously increasing the nitrogen content and decreasing the particle size of the substrate. No improvement occurred when these conditions were applied to cassava, however, because of the sticky consistency of the cassava after gelatinization.

INTRODUCTION

Mitchell et al. (1986) developed a model solid substrate to simplify studies of basic aspects of solid-state fermentation (SSF). This substrate consists of starch and other nutrients embedded in a kappa-carrageenan gel matrix. The model system was designed to mimic the SSF of chipped cassava tubers by Rhizopus oligosporus, a process which could be used for protein enrichment of cassava for use as an animal feed.

A comparison of growth of R. oligosporus on the model substrate and on cassava showed that growth was slower and final protein enrichment was poorer on the model substrate (Mitchell et al., 1986). Despite this, the model substrate has features which make it suitable for basic studies of SSF, namely that it is homogeneous and can be nutritionally defined. In addition, the mycelium can be recovered from the fermenting substrate after dissolving the carrageenan and digesting the starch, although biomass recovery is incomplete (Mitchell et al., 1986).

The present paper describes studies aimed at improving growth of

R. oligosporus on the model substrate. Results obtained with the model substrate were then applied to improvement of growth on cassava.

MATERIALS AND METHODS

Microorganism and maintenance, inoculum preparation, model and cassava substrate preparation, fermentation, sampling and assay procedures were done as described by Mitchell et al. (1986), with modifications as noted.

RESULTS AND DISCUSSION

Table 1 shows the modifications made to the model substrate of Mitchell et al. (1986) and the resulting protein enrichments. In no case was growth of Rhizopus oligosporus stimulated to the level obtained on the July 1985 cassava crop (CASS-85), which was the crop used by Mitchell et al. (1986). Growth on the July 1986 cassava crop (CASS-86) was significantly poorer than growth on the earlier crop. The protein enrichments on both crops were reproducible. Daubresse et al. (1987) noted that variation can occur in the composition of cassava due to the particular variety used and the growth and storage conditions.

Growth on the model substrate was not improved by the addition of yeast extract (CS-YE), casein hydrolysate (CS-CH), corn steep liquor (CS-CSL) or vitamins (CS-V), indicating that organic growth factors were not limiting. Addition of metal ions (MZ and CS-HTEF) stimulated protein production. Further, either cassava flour (CF) or a soluble cassava extract (CSEX) improved growth. The stimulatory nutrient could be magnesium. Daubresse et al. (1987) showed the importance of magnesium in SSF of cassava by Rhizopus oryzae.

Neither an increased buffering capacity (CSB) nor increasing the proportion of urea (CSU) improved growth. Therefore, acidification during growth is not a problem. This is due to the presence of urea, the hydrolysis of which produces ammonia which causes the pH to rise (Raimbault & Alazard, 1980). In fact growth was poorer with the increased proportion of urea, probably due to an increase in pH to unfavorable values.

Doubling either the nitrogen concentration (CS-2N) or the starch

Table 1. Effect of various substrate modifications on growth of Rhizopus oligosporus on the model substrate.

Medium	Enrichment ±95% confidence limits	Medium	Enrichment ±95% confidence limits
Effect of nutrient additions and modifications		Effect of nitrogen and size in combination*	
CS	8.8 ± 1.0	MZ-6mm	10.6 ± 1.3
CASS-85	20.6 ± 3.7	MZ-2mm	11.6 ± 1.0
CASS-86	14.2 ± 3.2	MZ-2N-6mm	12.7 ± 1.6
CS-YE	7.2 ± 4.1	MZ-2N-2mm	18.4 ± 1.8
CS-CH	7.2 ± 2.1		
CS-CSL	8.4 ± 2.7	Effect of inoculum density¢	
CS-V	8.5 ± 1.4		
MZ	10.6 ± 0.6	MZ-$4x10^6$	10.4 ± 0.9
CS-HTEF	12.2 ± 2.3	MZ-$3x10^5$	7.3 ± 1.1
CF	11.2 ± 3.1	MZ-$3x10^4$	5.0 ± 0.4
CSEX	11.9 ± 2.0		
CSB	7.2 ± 1.9	Effect of pregermination time#	
CSU	4.9 ± 0.5		
CS-2S	10.7 ± 0.9	MZ-0h	5.9 ± 0.5
CS-2N	10.0 ± 1.0	MZ-6h	7.3 ± 1.1
		MZ-12h	7.9 ± 3.0

Key: CS = 25 g cassava starch, 4 g kappa-carrageenan, 1.5 g $(NH_4)_2SO_4$, 0.5 g urea, 0.1 g K_2HPO_4, 0.1 g KH_2PO_4 and 100ml distilled water; 6mm cubes, 10^6 to 10^7 spores/ml in inoculum, inoculum 10% (v/w), incubated at 37 °C (Mitchell et al., 1986); CASS = cassava, variety MAUS VII harvested at the University of Queensland Agricultural Farm in July of the year indicated and prepared as described by Mitchell et al. (1986); YE = 0.5% yeast extract; CH = 0.5% casein hydrolysate; CSL = 0.5% corn steep liquor; V = 0.008% thiamin HCl, 0.006% folic acid, 0.008% calcium pantothenate, 0.003% B_{12}, 0.002% biotin, 0.002% p-aminobenzoic acid, 0.008% nicotinic acid and 0.006% pyridoxal phosphate; MZ = CS plus 0.1% $MgSO_4.7H_2O$ and 0.1% $ZnSO_4.7H_2O$; HTEF = 0.1% Hortico Trace Element Fertilizer; CF = CS but cassava starch replaced with cassava flour prepared by grinding dried tubers to pass a 1 mm sieve; CEX = CS but distilled water first homogenized with an equal weight of tuber and solids then removed by filtration; CSB = CS but the concentrations of buffer components increased 10-fold; CSU = CS but with the N content changed to 1% $(NH_4)_2SO_4$ plus 1% urea; CS-2S = CS but with starch concentration doubled; CS-2N = CS but with each N component increased 2-fold.

*Particle size and doubled N concentration specified.

¢Pregerminated 6 h, spore concentration in inoculum specified.

#Spore concentration in inoculum $3x10^5$ spores/ml, pregermination time specified.

concentration (CS-2S) improved growth slightly, indicating that diffusion may be limiting the rate of growth. With higher concentrations more substrate is available close to the surface and diffusion occurs over a shorter distance.

MZ was chosen as a suitable compostion for the model substrate because it stimulated growth and was defined, whereas all the other stimulatory supplementary nutrients were not. Table 1 shows that, in general, defined media gave more reproducible protein enrichments than undefined media. A further disadvantage of cassava flour and Hortico Trace Element Fertilizer was the presence of insoluble residues, which would interfere with the biomass recovery procedure of Mitchell et al. (1986).

Therefore both nutrition and diffusion affect growth of R. oligosporus in SSF. If diffusion is limiting then a smaller particle size should increase the rate of growth, by decreasing the distance over which diffusion occurs. The surface area to volume ratio will also increase, allowing more mycelial development before overcrowding halts growth. At a small enough particle size the growth rate should be only minimally affected by diffusion and exhaustion of the limiting nutrient should be the factor which halts growth.

MZ medium was used to compare two substrate particle sizes, 2 mm and 6 mm cubes. For each particle size cubes were prepared with the usual nitrogen content and with the nitrogen content doubled. The results are shown in Table 1. As previously observed, at the 6 mm particle size the higher nitrogen content (MZ-2N-6mm) improved growth slightly. Also, a decrease in particle size at the usual nitrogen content (MZ-2mm) had little stimulatory effect. However, a combination of smaller particle size and higher nitrogen content (MZ-2N-2mm) markedly improved growth; indicating that in the original model substrate (MZ-6mm) the nitrogen content and the available surface area were approximately equally limiting.

Inoculum preparation can be an important factor in SSF (Kronenberg, 1984). Both inoculum density and time of germination prior to inoculation were varied. Increases in both led to increased protein enrichment with inoculum density having the greater influence (Table 1).

The results suggested that growth would be best with a new model substrate (MAX) consisting of 50 g cassava starch, 6 g ammonium sulfate, 2 g urea, 0.1 g potassium dihydrogen phosphate, 0.1 g di-potassium hydrogen phosphate and 0.1 g Hortico Trace Element Fertilizer per 100 ml distilled water. The substrate was mashed to a particle size of 1 to 2 mm and the inoculum density was increased 10-fold over that used by Mitchell et al. (1986). Fig. 1 shows the growth obtained on MAX compared with growth on MZ-2N mashed to a similar particle size. Early growth on the two substrates was similar but growth on the mashed MZ-2N ceased at 24 h whereas growth on MAX continued to 36 h. The protein enrichment on MAX of 22.75 mg protein per gram initial substrate was the best enrichment yet obtained on the model substrate. The small particle size enabled 75% of the starch to be utilized within 36 h.

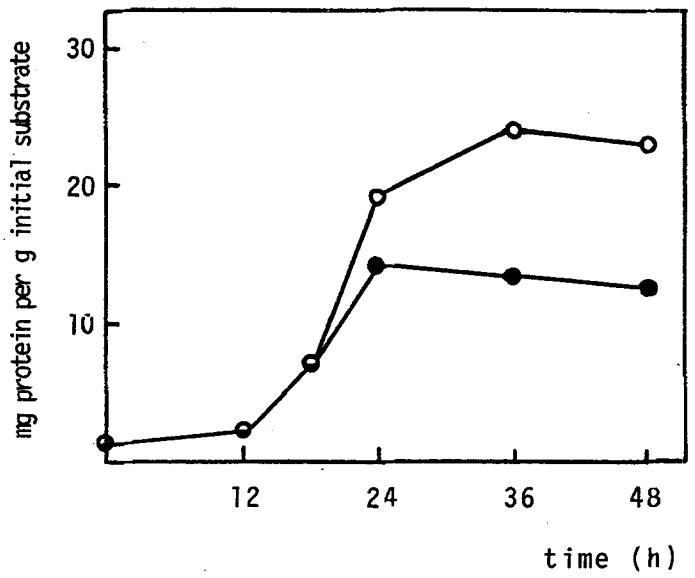

Fig. 1. Comparison of growth of R. oligosporus on (o) MAX (composition in text); and (•) mashed MZ-2N (composition in Table 1).

The results obtained on the model substrate should apply to cassava, enabling improved protein enrichment under similar conditions. A mashed cassava substrate was prepared by the method of Mitchell et al. (1986) except that the concentration of nitrogenous components was doubled, the gelatinization solution was supplemented with 0.3% (w/v) Hortico Trace Element Fertilizer and the tuber was mashed with a mortar and pestle prior to gelatinization. Growth was poorer on this

mashed cassava than on chipped cassava prepared by the method of Mitchell et al. (1986), as shown in Fig. 2. The mashed cassava became very sticky after gelatinization and the particles formed a large clump, decreasing the accessible surface area. Therefore, substrate consistency is just as important as particle size.

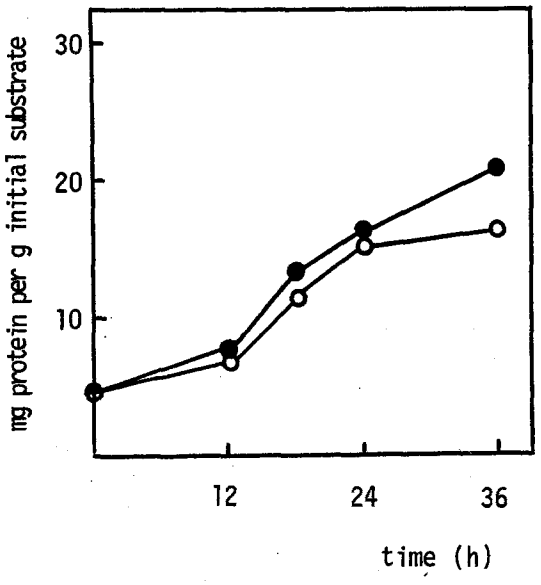

Fig. 2. Comparison of growth of R. oligosporus on (•) chipped cassava prepared by the method of Mitchell et al. (1986); and (o) mashed cassava (see text).

In conclusion, nutrition, diffusion, and particle size and consistency are parameters influencing the growth of Rhizopus oligosporus in SSF. Further optimization of nutrition should be done by response surface mapping. The problem of diffusion in SSF is poorly researched and deserves attention.

REFERENCES

Daubresse, P., Ntibashirwa, S., Gheysen, A. & Meyer, J.A. (1987). Biotechnology and Bioengineering 29, 962-968.

Kronenberg, H.J. (1984). Economic Botany 38, 433-438.

Mitchell, D.A., Greenfield, P.F. & Doelle, H.W. (1986). Biotechnology Letters 8, 827-832.

Raimbault, M. & Alazard, D. (1980). European Journal of Applied Microbiology and Biotechnology 9, 199-209.