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The scale up of mycelial shake flask fermentations: a case study of gamma linolenic acid production by *Mucor hiemalis* IRL 51

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SUMMARY

Production of gamma linolenic acid (GLA) by the filamentous fungus *Mucor hiemalis* IRL 51 was studied in both shake flask culture and in a 10-L stirred tank fermenter. This study was conducted to assess how the results from shake flask media screening trials compared to those obtained in a 10-L stirred tank fermenter, which is assumed to be more representative of an industrial system. The results show that the biological performance in 10-L fermenters is usually the same as that in shake flask culture. There were some inconsistencies which could possibly be attributed to scale, but no large differences were systematically seen. These results show that for this filamentous fungus, shake flask culture provides a quick and inexpensive way of optimizing medium composition.

INTRODUCTION

When designing an industrial fermentation medium, a large number of potential fermentation substrates are available for selection [8,18]. Each of these medium components could potentially have a beneficial effect on microorganism performance or on the medium cost/unit volumetric productivity. The only way to test this is in a set of medium optimization experiments. The strategy used for medium optimization can be relatively simple, for example changing one medium component concentration at a time [12], factorial experiments, or partial factorial experiments [15]; or it may involve more sophisticated methods, for example response surface methodology [16] or neural networks [9]. No matter which medium optimization strategy is chosen, a large number of experiments is needed. It is only practical to do these experiments in shake flask culture, because a large number of flasks can fit on a rotary or orbital shaker.

Several questions then arise. How comparable are the results from shake flasks to scaled-up higher volumes? Could the results achieved in a stirred tank 10-L fermenter be very different to those obtained from shake flask culture? The huge amount of data from shake flask cultures appearing in the literature makes these questions significant.

Most researchers fall into one of two schools of thought. The first (anti-shake flask school) does not think that shake flask characterization of microorganism performance or optimizations conducted in shake flasks have much relevance. These researchers often quote that the pH is not controlled in shake flasks [13], that the oxygen transfer capabilities of the shake flask is poor [6], that considerable evaporation takes place during shake flask culture, and that shake flask cultures lack adequate mixing [21]. One of the better descriptions of the disadvantages of shake flask culture is given by Solomons [19] who summarises 'the limitations of the system are considerable'.

The problem is compounded even further when mycelial organisms are grown. The anti-shake flask school states that the morphology of the organism can be different in a shake flask when compared to the morphology in stirred tank fermenters, and that this difference alone makes shake flask studies of little use. For example, the fungus may be in filamentous form in shake flask culture, but in single cell form in a stirred tank. Ratledge [17] screened ~300 organisms for GLA production in shake flask culture, and then took 80 of these organisms to be grown in 1-L submerged culture, presumably because of the limitations of shake flasks. Totani [20], studying arachidonic acid production by *Mortierella* reported on various parameters that affect the morphology of *Mortierella* when in shake flask and in a 4-L stirred fermenter.

The second (pro-shake flask) school, argues that there is no other way to do medium optimization apart from shake flasks simply because the number of experiments to be conducted is very large. It reasons that the effect of the different medium components is relative, and therefore the best medium in shake flask culture will also be the best medium in the stirred tank. For example, Beavan et al. [2] screened 6725 yeasts for oil content, with the aim of producing a cocoa butter equivalent, and finally reduced the

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list to three after small scale cultivation. Studies such as these reinforce the belief that what is done on a small scale will have relevance on a larger scale.

With mycelial organisms, the pro-shake flask school asserts that the oxygen demand during a typical fungal fermentation will be of the order of 10 mmol L^{-1} h^{-1}. For example Davies [3] quoted the specific oxygen consumption rate of oleaginous yeasts to be 2 mmol g^{-1} h^{-1}, which for a 20 g L^{-1} cell concentration gives an oxygen demand of 40 mmol L^{-1} h^{-1}. Studies in our laboratory at the 200-L scale with *M. hiemalis* IRL 51 indicate that the culture has a maximum oxygen uptake rate of 10 mmol L^{-1} h^{-1}. This is well below the level of up to 50–100 mmol L^{-1} h^{-1} that can be attained in shake flasks [19]. Whether the morphology of the organism is different, or whether this difference matters, depends on the organism under study. Finkelstein and Ball [4] state, 'Although shake-flask fermentations are relatively crude and physically unrelated to stirred vessels, they have proven to be quite valuable'.

Thus the literature offers both points of view. Shake flasks give substantially higher levels of polygalacturonase production by *Byssochlamys fulva* compared to a stirred fermenter [11], while levels of α-mannanase are much higher in stirred bioreactors than in shake flasks [14]. In many applications, sufficient oxygen supply may not be possible in conventionally shaped shake flasks [6], but very high aeration rates can be obtained with some shake flask designs [21].

This paper addresses the problem of the relevance of shake flask studies of filamentous microorganisms by studying the production of gamma linolenic acid (GLA) by *M. hiemalis*. This model system was chosen because: a) *M. hiemalis* is a filamentous fungus, b) gamma linolenic acid is a commercially significant pharmaceutical used in the health and nutrition field, and c) gamma linolenic acid production is currently the subject of a large amount of research [7]. This model system is also significant because there is a large amount of data taken from shake flasks on polyunsaturated fatty acid (PUFA) production by filamentous microorganisms [1,5].

In this study we employed a number of cultures using the same medium in both shake flask and 10-L stirred tank fermenters. These experiments were repeated with six different media and the results compared statistically.

MATERIALS AND METHODS

Organism

The organism studied was a GLA-producing fungus, *Mucor hiemalis* IRL 51 from the New Zealand Institute for Industrial Research and Development Culture Collection.

Media

Medium A: glucose 40 g L^{-1}, yeast extract 1 g L^{-1}, casein hydrolyzate 10 g L^{-1}. (Carbon:Nitrogen mole ratio = 18.7:1)
Medium B: glucose 40 g L^{-1}, yeast extract 10 g L^{-1}. (C:N = 27.0:1)
Medium C: glucose 40 g L^{-1}, peptone 5 g L^{-1}, yeast extract 1 g L^{-1}. (C:N = 27.1:1)
Medium D: glucose 20 g L^{-1}, raw sugar 20 g L^{-1}, peptone 10 g L^{-1}, yeast extract 1 g L^{-1}. (C:N = 16.3:1)
Medium E: glucose 200 g L^{-1}, yeast extract 1 g L^{-1}, casein hydrolyzate 10 g L^{-1}. (C:N = 76.1:1)
Medium F: molasses 80 g L^{-1}, peptone 15 g L^{-1}, yeast extract 15 g L^{-1} (C:N = 9.8:1)

Tap water was used to make up the media. Raw sugar was a commercially available sucrose preparation.

Growth conditions

Shake flask cultures were grown in baffled 500-ml flasks containing 150 ml of culture medium. The cultures were grown for 6 days at 25 °C on a rotary shaker at 140 r.p.m. The 10-L cultures were grown for 6 days at 25 °C in a stirred tank fermenter with two Rushton impellors, rotating at 600 r.p.m. with an air flow rate of 1 v.v.m. (volume/volume/minute). The 10-L fermenter had a working volume of 8 L. The only major difference between the shake flask and the 10-L fermenter was pH control. In shake flasks the pH was initially adjusted to 6.5–7.0 with no pH control during the fermentation, whereas in the 10-L fermenter cultures pH was controlled at 5, an average pH encountered during shake flask culture.

Assays

Three different assays were performed on the fungus; dry cell weight, oil content of the cell, and fatty acid content of the oil. Cell dry weight was measured by taking a known wet weight of mycelium containing fermentation broth, vacuum filtering, washing and drying it at 70 °C overnight to constant weight, and then reweighing it. Oil content was measured by initially extracting the biomass with chloroform:methanol (2:1 v/v) and then ethanol:hexane (1.5:1 v/v). The solvent from both extractions was then evaporated and the oils pooled. The pooled oil was dissolved in hexane, the undissolved biomass separated, and the hexane evaporated to yield the oil. This was weighed to give the oil content of the original biomass. The fatty acid content of the oil was obtained by converting the fatty acids in the oils to fatty acid methyl esters (FAMES). These FAMES were then analyzed by gas chromatography. A detailed description of all the methods used can be found in Kennedy et al. [10].

RESULTS AND DISCUSSION

The data for comparing results from shake flasks with those obtained from 10-L stirred tank fermenters are distributed over the six different media (denoted A–F). For medium A, we used nine replicates for shake flask experiments and four replicates for the 10-L stirred tank fermenter. For each of the other media there are results from two shake flasks, except for medium F for which there are three, and one 10-L stirred tank fermenter result. The data are shown in Table 1.

The data were analyzed in two different ways. First, statistical comparisons between the two fermentation bioreactor types were conducted on the results of medium A in

TABLE 1

Results for the growth of *M. hiemalis* IRL 51 in shake flasks and 10-L fermenters

Media	Results from:					
	Shake flasks			10-L Fermenters		
	Cell conc. ($g\ L^{-1}$)	Oil content (%)	GLA content (%)	Cell conc. ($g\ L^{-1}$)	Oil content (%)	GLA content (%)
A	16.5	18.4	10.6	17.6	29.0	9.8
	16.4	17.9	10.8	20.7	29.7	9.0
	15.2	18.2	11.2	16.1	11.6	7.0
	16.4	19.2	10.4	17.8	16.4	7.3
	15.6	18.9	11.2			
	16.0	22.0	10.8			
	18.4	26.0	9.4			
	16.4	26.4	7.8			
	17.4	25.1	10.0			
B	11.2	10.8	9.8	18.6	20.4	9.5
	14.8	14.9	8.6			
C	11.6	33.9	7.9	8.4	33.0	7.5
	11.5	31.7	8.0			
D	9.2	16.0	10.7	13.3	17.8	9.2
	9.9	16.1	10.7			
E	17.8	35.1	3.7	18.2	12.0	3.8
	10.6	28.2	2.7			
F	10.2	4.4	15.8	14.3	7.0	20.0
	10.7	3.7	16.0			
	11.4	3.3	15.7			

isolation. The second statistical analysis included all the media combined, to judge the effect of medium on the results. For results obtained with medium A, a two sample *t*-test was carried out. The results are given in Table 2. The only significant difference ($P < 0.05$) being for GLA, in which the shake flask gave a mean 24% higher than the 10-L stirred tank fermenter.

Considering all the media, a regression model was fitted to the data as follows:

$$y_{ijk} = \mu_i + \alpha_j + e_{ijk}$$

where: y = cell ($g\ L^{-1}$), oil (%) or GLA (%)
i = medium (A–F)
j = shake flask fermenter or 10-L stirred tank fermenter
k = replicate
μ_i = mean for medium i
α_j = overall effect of fermenter j

TABLE 2

Comparison of shake flask and 10-L stirred tank fermentation means *for medium A* (SF = shake flask, 10 L = 10-L stirred tank fermenter, df = degrees of freedom, P = probability)

Variable	Mean		Difference (SF−10 L)	Std error of difference	t(11 df) * = $P<0.05$
	10 L	Shake flask			
Cells ($g\ L^{-1}$)	18.05	16.48	−1.57	0.78	−2.03
Oil (%)	21.68	21.35	−0.33	3.39	−0.10
GLA (%)	8.27	10.24	1.97	0.70	2.83*

* Significant difference at the 95% probability level.

TABLE 3

Comparison of shake flask and 10-L stirred tank fermentation means *over all media* (SF = shake flask, 10 L = 10-L stirred tank fermenter, df = degrees of freedom, P = probability)

Variable	Mean		Difference (SF−10 L)	Std error of difference	t(22 df) * = $P<0.05$
	10 L	Shake flask			
Cells ($g\ L^{-1}$)	16.10	13.87	−2.23	0.79	−2.83*
Oil (%)	19.17	19.73	0.56	2.25	0.25
GLA (%)	9.49	9.98	0.49	0.54	0.90

* Significant difference at the 95% probability level.

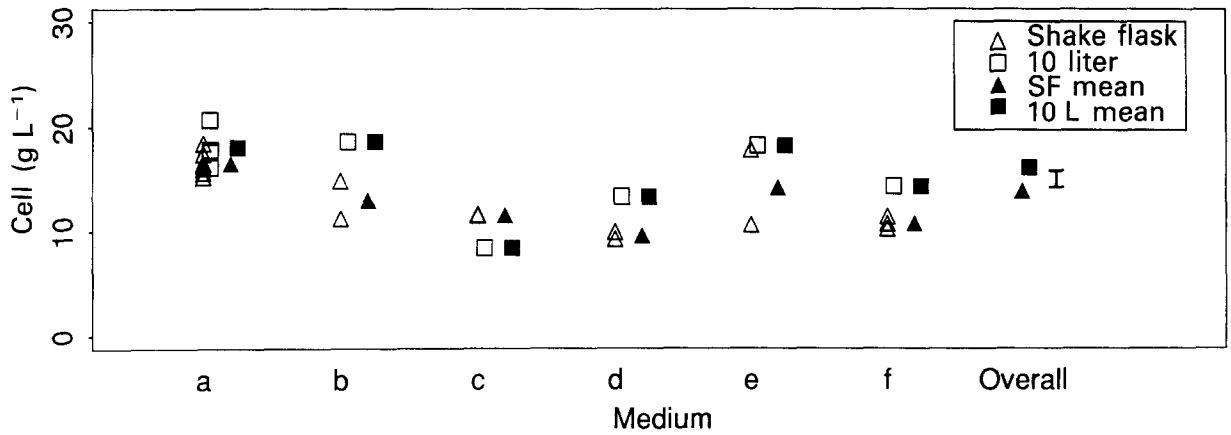

Fig. 1. Individual samples, means for each medium, and weighted mean over all media, *for the cell concentration*, shake flask and 10-L stirred tank data. The error bars show least significant difference (P = 0.05) of the overall means for the variate.

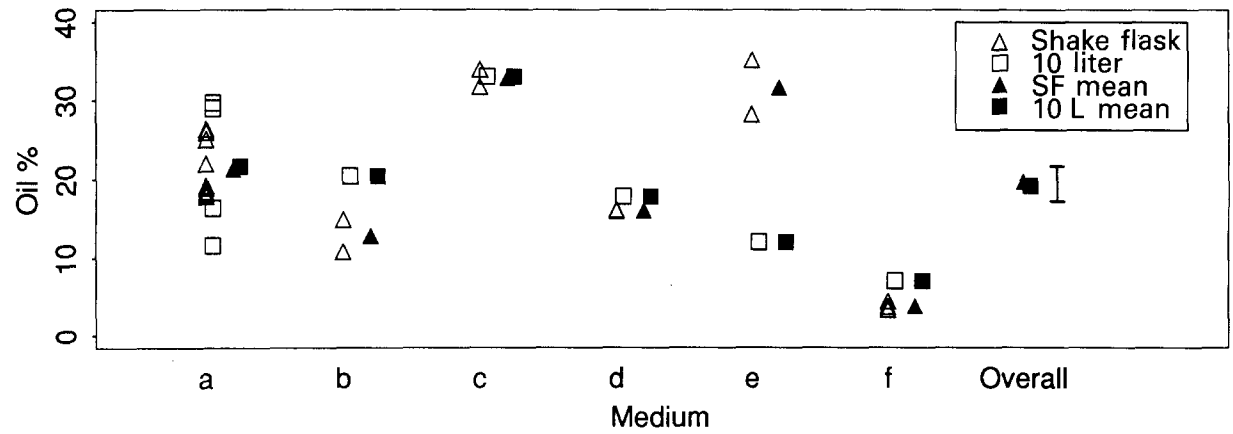

Fig. 2. Individual samples, means for each medium, and weighted mean over all media, *for the oil content*, shake flask and 10-L stirred tank data. The error bars show least significant difference (P = 0.05) of the overall means for the variate.

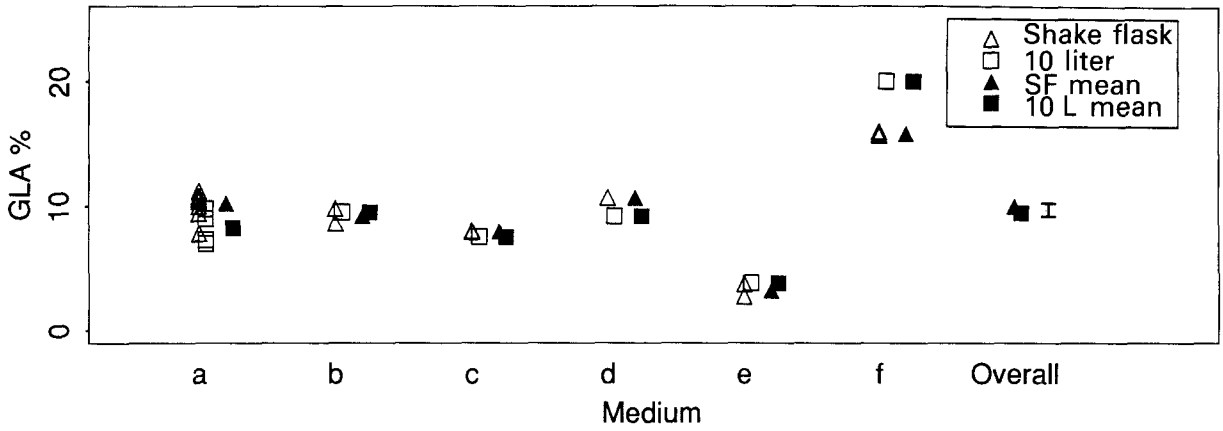

Fig. 3. Individual samples, means for each medium, and weighted mean over all media, *for the GLA content*, shake flask and 10-L stirred tank data. The error bars show least significant difference (P = 0.05) of the overall means for the variate.

e = random error.

Because of the small number of data, no term was fitted for the interaction between medium and fermenter effects. The quality of interest is $\alpha_{10\,L} - \alpha_{SF}$, the overall difference between the shake flask and 10-L stirred tank fermenter means. The results of tests of significance on this difference are given in Table 3. The only statistically significant difference (P<0.05) being for cell concentration, in which

the shake flask gave a mean 14% lower than the mean from the 10-L stirred tank fermenter.

Figures 1–3 show results for individual samples, means for each type of bioreactor for each medium, the overall mean for each bioreactor type, and the least significant difference (LSD) for the overall means. The means over all media were obtained by weighting the mean for each medium in proportion to the marginal total number of observations (10 L + SF) for that medium.

While more data would have been desirable, these results show that the biological performance in 10-L fermenters is usually the same as that in shake flask culture. There were some inconsistencies which could be attributed to scale, but no large differences were systematically seen. Thus, although confirmatory fermentations should be performed for a specific fermentation by the industrial microbiologist scaling up shake flask data, shake flask fermentations are still a very useful and important tool for media design and optimization.

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