Appl Microbiol Biotechnol (1987) 26:456–461

Applied and Microbiology Biotechnology

Ergosterol content of *Rhizopus oligosporus* NRRL 5905 grown in liquid and solid substrates

M. J. R. Nout[1], T. M. G. Bonants-van Laarhoven[1], P. de Jongh[1], and P. G. de Koster[2]

[1] Department of Food Science, Agricultural University, and
[2] DMV Campina, Veghel, The Netherlands

Summary. The ergosterol content of *Rhizopus oligosporus* NRRL 5905 varied between 2—24 μg/mg biomass dry matter when grown in laboratory media and was found to be influenced by the substrate composition.

When grown on a natural substrate (soya beans) the ergosterol content was considerably higher (estimated at approx. 60—90 μg/mg biomass dry matter).

In laboratory media, the ergosterol content was also influenced by the extent of aeration and the growth phase of the mycelium; within the range of 25° C—35° C, the incubation temperature did not influence the ergosterol content significantly.

In view of these variations, ergosterol should not be used as a chemical index for the quantification of biomass grown in static solid-substrate fermentations with limited mass transfer, e.g. tempe or oncom.

Introduction

The industrial importance of solid-substrate fermentations (SSF) as applied in the oriental-food industry, manufacture of mould-ripened cheeses, composting and spawn production was mentioned by Cannel and Moo-Young (1980a, b) and Matcham et al. (1985).

The optimisation and process control of fermentation conditions require reliable and preferably rapid methods for the determination of microbial biomass.

Offprint requests to: M. J. R. Nout, Department of Food Science, Agricultural University, De Dreijen 12, 6703 BC Wageningen, The Netherlands

Due to mycelium penetration and attachment to the substrate, techniques for bacterial or yeast biomass determination (Harris and Kell 1985) are not always suitable in the case of fungal SSF.

Enumeration of fungal propagules is subject to high coefficients of variation and reflects a degree of sporulation rather than biomass (Jarvis et al. 1983; Nout 1984).

Specific attempts to monitor fungal biomass in SSF's have been made with e.g. the asbestos-koji method (Sakaguchi et al. 1955), nylon-paste method (Yamamoto 1957), enzymatic degradation of the substrate and subsequent collection of biomass by filtration (Charles and Gavin 1977), measuring the texture of the fermented substrate (Kronenberg and Hang 1985), measuring CO_2 and heat production (Rathbun and Shuler 1983; Finger et al. 1976), determination of the chitin content of the fermented substrate (Arima and Uozumi 1967; Cousin et al. 1984; Matcham et al. 1985) and by determination of the laccase activity during the fermentation (Matcham et al. 1985).

The latter also compared the chitin and laccase methods with the determination of ergosterol, the predominant sterol in fungi. They observed a good correlation between the biomass of *Agaricus bisporus* grown on liquid medium and the total ergosterol yield, and found the ergosterol content a more sensitive chemical index for low levels of mycelial growth than chitin or laccase activity.

Based on an ergosterol content of 2.2 μg/mg dry weight mycelium grown on liquid substrate, they predicted from the ergosterol content of colonised rye grain that it would contain 10 mg dry weight mycelium per g dry weight grain.

Since the determination of ergosterol by GLC or direct UV-spectroscopy as carried out by Matcham et al. (1985) was not entirely satisfactory,

they recommended the use of the HPLC-method as employed by Seitz et al. (1977). Our interest in the tempe fermentation prompted us to investigate the possible use of ergosterol as a chemical index for the quantification of *Rhizopus oligosporus* biomass growing on liquid and solid substrates. Our particular interest was the predictability of the ergosterol content of the fungal fermented material under varying conditions of age and culturing conditions.

Materials and methods

Organism

Rhizopus oligosporus NRRL 5905, classified by Schipper and Stalpers (1984) as *Rhizopus microsporus* var. *oligosporus*, was grown at 30° C and maintained on malt extract agar (Oxoid, CM 59).

Spore suspension

R. oligosporus was grown on mung bean sprout agar for 1 week at 30° C to achieve dense sporulation. Mung bean sprout agar contained 60 g sucrose, 20 g agar and 1000 g mung bean sprout extract, obtained by boiling 100 g mung bean sprouts *(Vigna radiata)* in 1 l dist. water for 30 min, followed by filtration and adjusting to 1000 g with dist. water. The sporangia were scraped from the agar and suspended in sterile dist. water + 1% Tween 80, collected by centrifugation at 3000 rpm for 15 min, washed and suspended in sterile dist. water to obtain a suspension containing $5 \cdot 10^5$—$3 \cdot 10^6$ viable spores/ml. This suspension could be stored at 4° C for 7 days without significant loss of viability.

Liquid cultures

Conical flasks (250 ml size) containing 100 ml sterile Tryptone Soya broth (TSB, Oxoid CM 129), TSB + 2% glucose (TSBG), or malt extract broth (MEB, Oxoid CM 57) were inoculated with approx. 10^5 viable spores/ml and incubated either stationary or in a temperature controlled rotary shaker (120 rpm). Experiments at reduced oxygen concentration were carried out in an incubator permanently flushed with a mixture of 97% N_2 and 3% O_2.

Solid cultures

Experiments on agar medium were carried out with TSB solidified with 1.2% agar in 14.5 cm diam. petri dishes, inoculated by placing a drop of spore suspension containing approx. 10^4 viable spores at the centre of the plates.

Growth on soya beans was studied after they had been prepared for tempe manufacture, as follows: 1 kg of dry dehulled yellow var. soya beans were rinsed twice with tapwater, mixed with 3 l tapwater of 45° C, and soaked at 25° C for 18—24 h. After discarding the soakwater, the beans were rinsed once with tapwater, boiled in clean tapwater for 20—25 min, collected on a colander and allowed to cool and dry superficially, while spread in a layer of 1—2 cm thickness. After cooling to room temperature, the beans were inoculated with approx. 10^5 spores/g as mentioned by Ko and Hesseltine (1979), placed in a single layer in perforated petri dishes and incubated at 30° C.

Dry matter

Fungal dry matter in liquid medium was collected in a buchner funnel on a pre-weighed filterpaper (Schleicher and Schüll 520b), rinsed with dist. water, dried at 80° C for 24 h, cooled in a desiccator, and weighed.

Fungal dry matter on solid agar medium was removed, together with the agar medium, from the petri dish, placed in a glass beaker with 250 ml tapwater + 0.05% Tween 20, and heated in a microwave oven, causing the agar medium to melt and diffuse into the water. The fungal mass remained intact and was collected, rinsed, dried and weighed.

Fungal biomass grown on soya beans was estimated as follows: duplicate samples of soya bean cultures were homogenised with dist. water for 2 min (Colworth Stomacher, type 400) to give 1:5 dilutions. These were diluted with an equal volume of a 0.12% methylene blue solution to improve the contrast for microscopic viewing. A known weight of the resulting 1:10 dilution (approx. 0.06 g) was placed on duplicate microscope slideglasses, and covered with 18 × 18 mm cover slips. Alternatively, methylene blue was omitted and 1:10 dilutions in water were used for photographic purposes. Of each wet mount, 10 microscopic fields (magn. 135 ×) were randomly selected and photographed (Zeiss, Axiomat). The volume of the visible fungal mass was calculated from its estimated length and diameter, and compared with a series of similarly prepared mounts of increasing known weights of fungal mass pre-grown in liquid substrate and suspended in sterile soya beans, using a Waring blender.

Ergosterol

An amount of 0.1 g of comminuted and lyophilised mycelium sample or 0.5—1 g of a similarly prepared sample of soya bean culture was homogenised for 2 min with 25 ml abs. ethanol pro analysis using an Ultra Turrax blender.

The slurry was transferred into a 250 ml round-bottom flask and 25 ml 2 N ethanolic NaOH and 3 boiling chips were added. Saponification was carried out by refluxing for 1 h. After cooling, solids were removed by centrifugation for 20 min at 2500 rpm. The supernatant was transferred into a separatory funnel together with 50 ml dist. water. The pellet was resuspended in 10 ml petroleum ether (PE) b.p. 60°—80° C (techn. grade), centrifuged for 20 min at 2500 rpm, and the supernatant added to the saponified mixture in the separatory funnel. After shaking, the PE fraction was collected, and the watery phase was extracted with 3 × 50 ml PE b.p. 60°—80° C. The four combined PE fractions were evaporated to dryness in a vacuum rotary film evaporator (water bath temperature 40° C). Traces of water were removed by dissolving the residue in 10 ml acetone followed by evaporation to dryness as above. The residue was finally dissolved in 5 ml abs. ethanol.

After filtration through a 0.45 micron Millipore filter, aliquots of 20 µl were injected onto a 10 ODS Sphercolumn, 250 × 4.6 mm (Chrompack), protected by a Nucleosil (30—35 micron) pre-column (Waters), fitted in a SpectraPhysics SP 8000 HPLC with a variable wavelength detector at 282 nm (Kratos Spectraflow 773; bandwith 5 nm). Chromatographic conditions: mobile phase methanol:water (92/8 v.v.), flow 1.5

ml/min, column temperature 40° C, retention time ergosterol approx. 12 min. A standard solution of ergosterol (Sigma E 6625) in abs. ethanol was used for quantification purposes.

Results and discussion

Determination of ergosterol and its distribution

The frequently cited procedure of Seitz et al. (1977) involving chromatography of a saponified extract of the sample does not allow non-extractable ergosterol derivatives to be measured. Such membrane-bound ergosterol derivatives could be extracted however, after preliminary saponification of the sample. With the described method a recovery of 95% of ergosterol standard solutions was obtained.

Since a 20 hour exposure to diffuse daylight caused a loss of 1.6% of the ergosterol in a 50 mg/l solution, samples intended for storage longer than 1 hour were kept in the dark.

We obtained a coefficient of variation (c.v. $= \sigma/\mu \cdot 100\%$) of the ergosterol determination method of 3.0% (5 replicate analyses of 1 sample of *R. oligosporus* mycelium grown in TSB), an accuracy similar to that achieved by Delaveau et al. (1983). We obtained a c.v. of 15.7% for the ergosterol content of the fungal biomass, comparing favourably with that of the biomass itself, viz.: c.v. 30.4% (both calculated from single analyses of five replicate cultures).

Table 1 shows that in a liquid TSB culture, only 4% of all detected ergosterol was found to be excreted into the medium. For this reason and also to avoid having to lyophilise large volumes of liquid medium, we limited ourselves whenever possible to the ergosterol content of the fungal mass only. From the same table it shows that approx. 10% of the biomass ergosterol is derivatised,

Table 1. Distribution of ergosterol in *Rhizopus oligosporus* (24-hour TSB shaking culture at 30° C)

Medium:	4.0
Biomass:	
Ethanol extractable without prior saponification:	
Ergosterol	86.4
Ergosteryl esters (determined as ergosterol after their saponification)	2.2
Ethanol extractable only after saponification of biomass:	
Ergosteryl esters (determined as ergosterol)	7.4
Total ergosterol	100.0%

the majority of which is probably cellwall- or membrane-coupled and only to be extracted and detected after having been released by saponification of the biomass. A smaller quantity of ergosterol derivatives is ethanol soluble without prior saponification of the biomass, as well as the freely occurring ergosterol which constitutes the majority of total detectable ergosterol. Since we could not assume the ratio given in Table 1 to be constant, most samples of biomass were analysed for total ergosterol.

Ergosterol content of R. oligosporus biomass grown on defined growth media

Using defined growth media and conditions permitting the gravimetric determination of biomass dry weight, the influence of incubation time, composition of the substrate, culturing conditions, aeration and temperature were investigated as summarised in Fig. 1 A—D.

Seitz et al. (1979) compared chitin and ergosterol contents of fungal biomass grown on liquid malt extract media. *Alternaria alternata* contained 3.8—4.4 μg ergosterol/mg dry weight (5 and 10 days incubation, respectively), *Aspergillus flavus* 2.3—3.3 μg/mg (2.1 and 7 days, resp.) and *A. amstelodami* 5.9—4.9 μg/mg (3.6 and 8.6 days, resp.). Seitz and Pomeranz (1983) reported the ergosterol content of sclerotia of *Claviceps purpurea* of unstated age, removed from ergoty cereals and grasses, to vary between 0.2—0.83 μg/mg dry weight. Matcham et al. (1985) found ergosterol contents of 2—2.7 μg/mg dry weight in *Agaricus bisporus* between 21 and 56 days of incubation on a liquid medium. At 56 days the stationary phase had just been reached; no data were given beyond that stage.

Except for those given by Seitz and Pomeranz (1983) for *C. purpurea*, the above ergosterol contents are comparable to the ones found by us in *R. oligosporus* when grown in or on liquid media during the stage of rapid growth.

However, our results indicate that the ergosterol content of *R. oligosporus* biomass may become significantly higher as influenced by the composition of the substrate, the extent of aeration and the age of the fungal material. The tested incubation temperatures had no significant effect.

Weete et al. (1973) already mentioned the need for more data concerning the changes in total lipids and sterol contents during the fungal life cycle. The gradual increase of the ergosterol content found by us is most likely caused by the de-

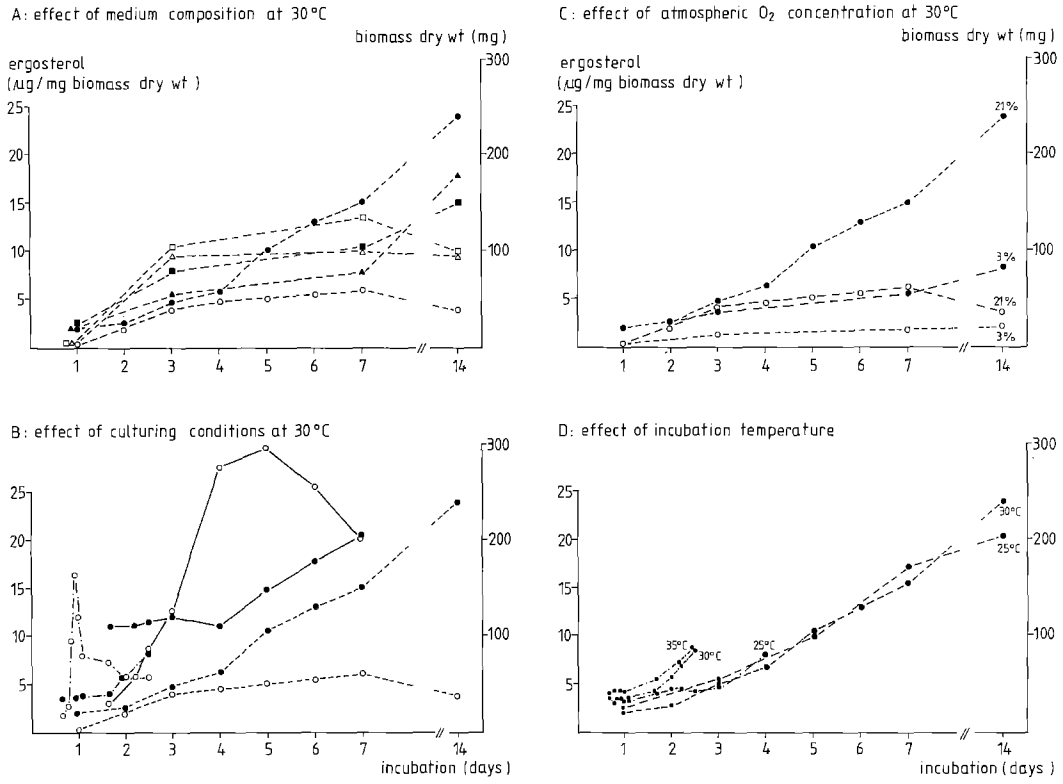

Fig. 1. Influence of environmental conditions on the yield of *Rhizopus oligosporus* biomass and its ergosterol content. TSB (tryptone soya broth or agar): ergosterol ●; biomass ○. TSB + 2% glucose: ergosterol ▲; biomass △. MEB (malt extract broth): ergosterol ■; biomass □. ——— Solid medium; ----- liquid medium (stationary culture); -·-·-·- liquid medium (shaking culture)

crease of the biomass dry weight due to senescence and death. The phenomenon of a sudden, almost linear increase of the ergosterol content beyond the stationary growth phase became particularly evident during the experiments on solid agar medium (Fig. 1B); a similar, albeit more gradual pattern emerges from the experiments in liquid cultures.

The influence of aeration was significant. Whereas well-aerated biomass of *R. oligosporus* grown on a solid agar surface had an initial ergosterol content of 11 µg/mg dry weight (Fig. 1B), the biomass grown in liquid medium at 3% O_2 contained only 2.1 µg/mg which remained significantly lower than that of liquid grown biomass at 21% O_2 (Fig. 1C). This effect appears similar to that observed in *Saccharomyces uvarum* by Delaveau et al. (1983) who reported that this yeast was unable to produce ergosterol under anaerobic conditions, and that the ergosterol in aerobically-grown yeast disappeared gradually during anaerobic growth.

The influence of oxygen availability on the ergosterol content may have important practical consequences if ergosterol were to be used as a chemical index for the quantification of biomass produced in static solid-substrate fermentations such as the tempe fermentation.

During tempe manufacture, a stationary packed bed of cooked soya beans is overgrown and penetrated by fungi, mainly *R. oligosporus*. Practical experience has shown (Ko and Hesseltine 1979) that the soya bean bed should not exceed approx. 5 cm thickness since otherwise mould growth would be impaired by lack of oxygen. Rathbun and Shuler (1983) found that *R. oligosporus* still showed relatively rapid growth at 1—6.5% O_2 but that no growth occurred at 0.23—0.32% O_2.

Assuming a bed of 5 cm thickness with air access from both sides, and a concentration of 1% O_2 in the centre (still permitting fungal growth), the O_2 gradient in fermenting tempe could be estimated at $(21-1\%)/2.5$ cm = 8% O_2/cm depth, approximately. Considering the effect of O_2 concentration on the ergosterol content of the biomass, it should therefore be expected that the ergosterol content of the biomass will decrease towards the

Table 2. Aerobic growth and biomass ergosterol content of *Rhizopus oligosporus* on soya beans at 30° C

Incubation period (h)	Estimated biomass		Ergosterol (μg/g tempe dry wt)	Estimated biomass ergosterol content (μg/mg biomass dry wt)
	fresh weight (%, w/w)	dry matter (mg/g tempe dry wt)		
24	1.0	4.5	420	93
48	5.8	27.2	1940	71
72	9.5	50.4	3230	64

centre of the bed during this and similar static SSF's, and consequently, that it will not be of much value as a chemical index for biomass.

The biomass ergosterol content may be more homogenous during agitated SSF's in rotating drum fermenters (Lindenfelser and Ciegler 1975) with controlled atmospheric conditions.

Ergosterol content of R. oligosporus biomass grown on soya beans

Table 2 presents data of *R. oligosporus* grown on soya beans. In order to minimise the effect of aeration, the beans were incubated as single layers in petri dishes under aerobic conditions.

The photomicroscopic method which we employed to estimate the quantity of biomass proved to be cumbersome. In addition, the accuracy of this method leaves much to be desired since its low recovery (20%—25%) requires considerable extrapolation of measured data. Nevertheless, the obtained estimates are comparable to those of Charles and Gavin (1977) who obtained 10% biomass during the rapid growth phase of *R. oligosporus* in tempe.

Since the soya beans themselves did not contain detectable amounts of ergosterol, and the predominant soya sterols β-sitosterol and stigmasterol do not absorb light of 282 nm, there is no doubt that the gradual increase of the ergosterol content of the fermenting beans is due to the fungal growth.

The estimated biomass ergosterol content in soya beans was of the order of 60—90 μg/mg biomass dry matter and underlines the strong effect of substrate composition as was illustrated in Fig. 1A.

During the experiments summarized in Table 2, the combined presence of (a) soya compounds (e.g. fatty acids) which can be incorporated into the ergosterol synthesis pathway, and (b) an unlimited supply of oxygen, resulted in biomass ergosterol contents which are approximately 10-fold higher than those reported in the literature (and confirmed by us) in defined liquid growth media.

Our data therefore rule out the possibility of predicting the biomass grown in natural substrates on the basis of the ergosterol content of biomass grown on defined laboratory media.

References

Arima K, Uozumi T (1967) A new method for the estimation of the mycelial weight in koji. Agric Biol Chem 31:119—123

Cannel E, Moo-Young M (1980a) Solid-state fermentation systems. Process Biochem 15:2, 4, 6—7

Cannel E, Moo-Young M (1980b) Solid-state fermentation systems. Process Biochem 15:24—28

Charles M, Gavin JR (1977) Engineering studies of the tempe fermentation. In: Steinkraus KH (ed) Handbook of indigenous fermented foods. Marcel Dekker, New York, pp 74—87

Cousin MA, Zeidler CS, Nelson PE (1984) Chemical detection of mold in processed foods. J Food Sci 49:439—445

Delaveau J, Lipus G, Moll M (1983) Importance des stérols au cours du cycle de la levure de brasserie. Bios 14:37—40

Finger SM, Hatch RT, Regan TM (1976) Aerobic microbial growth in semisolid matrices: heat and mass transfer limitation. Biotechnol Bioeng 18:1193—1218

Harris CM, Kell DB (1985) The estimation of microbial biomass. Biosensors 1:17—84

Jarvis B, Seiler DAL, Ould AJL, Williams AP (1983) Observations on the enumeration of moulds in food and feedingstuffs. J Appl Bact 55:325—336

Ko SD, Hesseltine CW (1979) Tempe and related foods. In: Rose AH (ed) Economic Microbiology, Vol. 4. Microbial Biomass. Academic Press, London, pp 115—140

Kronenberg H-J, Hang YD (1985) A puncture test method for monitoring solid substrate fermentation. J Food Sci 50:539—540

Lindenfelser LA, Ciegler A (1975) Solid-substrate fermentor for ochratoxin A production. Appl Microbiol 29:323—327

Matcham SE, Jordan BR, Wood DA (1985) Estimation of fungal biomass in a solid substrate by three independent methods. Appl Microbiol Biotechnol 21:108—112

Nout MJR (1984) Influence of sample size and analytical procedure on the variance of surface mould plate counts of maize kernels. Chem Mikrobiol Technol Lebensm 8:133—136

Rathbun BL, Shuler ML (1983) Heat and mass transfer effects in static solid-substrate fermentations: design of fermentation chambers. Biotechnol Bioeng 25:929—938

Sakaguchi K, Okazaki H, Takeuchi M (1955) A note on the comparison of koji and submerged cultures. J Agr Chem Soc Japan 29:349

Schipper MAA, Stalpers JA (1984) A revision of the genus Rhizopus. 2. The Rhizopus microsporus group. Studies in Mycology (Baarn) No. 25:20—34

Seitz LM, Mohr HE, Burroughs R, Sauer DB (1977) Ergosterol as an indicator of fungal invasion in grains. Cereal Chem 54:1207—1217

Seitz LM, Sauer DB, Burroughs R, Mohr HE, Hubbard JD (1979) Ergosterol as a measure of fungal growth. Phytopath 69:1202—1203

Seitz LM, Pomeranz Y (1983) Ergosterol, ergosta-4,6,8(14),22-tetraen-3-one, ergosterol peroxide, and chitin in ergoty barley, rye and other grasses. J Agric Food Chem 31:1036—1038

Weete JD, Lawler GC, Laseter JL (1973) Total lipid and sterol components of Rhizopus arrhizus: identification and metabolism. Arch Biochem Biophys 155:411—419

Yamamoto K (1957) Studies on koji. 1. A new analytical method of koji. Bull Agr Chem Soc Japan 21:308—312

Received February 23, 1987/Revised April 15, 1987