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Towards the design of an optimal strategy for the production of ergosterol from *Saccharomyces cerevisiae* yeasts

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Abstract

The total yield of ergosterol produced by the fermentation of the yeast *Saccharomyces cerevisiae* depends on the final amount of yeast biomass and the ergosterol content in the cells. At the same time ergosterol purity - defined as percentage of ergosterol in the total sterols in the yeast - is equally important for efficient downstream processing. This study investigated the development of both the ergosterol content and ergosterol purity in different physiological (metabolic) states of the microorganism *S. cerevisiae* with the aim of reaching maximal ergosterol productivity. To expose the yeast culture to different physiological states during fermentation an on-line inference of the current physiological state of the culture was used. The results achieved made it possible to design a new production strategy, which consists of two preferable metabolic states, oxidative-fermentative growth on glucose followed by oxidative growth on glucose and ethanol simultaneously. Experimental application of this strategy achieved a value of the total efficiency of ergosterol production (defined as product of ergosterol yield coefficient and volumetric productivity), 103.84×10^{-6} $gL^{-1}h^{-1}$, more than three times higher than with standard baker's yeast fed-batch cultivations, which attained in average 32.14×10^{-6} $gL^{-1}h^{-1}$. At the same time the final content of ergosterol in dry biomass was 2.43 %, with a purity 86 %. These results make the product obtained by the proposed control strategy suitable for effective down-stream processing.

Keywords: Bioprocess control; *Saccharomyces cerevisiae* yeast; Ergosterol production; Physiological control; Knowledge-based control

1 Introduction

Sterols are essential structural and regulatory components of eukaryotic cell membranes.[1] The predominant sterol in yeasts, ergosterol, is known as provitamin D2 and is the main precursor of cortisone and the hormone progesterone. Also discovered recently is its potential role in the development of anticancer drugs.[2]

As an economically important product ergosterol is produced by the fermentation of strains of the yeast *Saccharomyces cerevisiae*. Methods for trying to increase the ergosterol productivity of yeast strains are therefore of great interest. These methods are generally based on two approaches: the engineering of metabolic pathways,[3] and the physiological approach exploiting knowledge about the physiology of the yeast strain and its response to changing environmental conditions.[4]

Concerning the first approach the lipid pathways in this organism have been described in detail and are well characterized.[5] Wild types of *S. cerevisiae*, the metabolism of which has been frequently studied, have demonstrated a very similar sequence of reactions leading to ergosterol.[6] While ergosterol itself is a main regulator of the ergosterol biosynthetic pathway, several steps in this pathway are also regulated at the transcriptional level. Moreover, regulation is also greatly dependent on heme.[7,8] An additional important regulatory mechanism is the reversible esterification of sterols, by which most of the sterol intermediates and ergosterol itself can be esterified and stored as steryl esters in lipid particles of yeast.[9,10] A coordinated regulation of several lipid biosynthetic pathways (sterols, sphingolipids, fatty acids) also influences ergosterol synthesis.[11-13]

Metabolic engineering is used also to improve ethanol and ergosterol co-production.[14-16] Genetic manipulation of *S. cerevisiae* to get a strain with higher thermotolerance for effective ethanol production has been carried out.[17] These authors concluded that the shift to higher thermotolerance resulted from the substitution of ergosterol by fecosterol which is responsible for optimal membrane fluidity.

The production of ergosterol by a recombinant *S. cerevisiae* strain with an increased capacity for sterol formation using cane molasses as an inexpensive carbon source has been investigated.[18,19]

Similar studies have also been done with such substrates as modified corn straw hydrolysate[20] or corn straw hydrolysate and soybean meal liquor.[21]

The production of ergosterol by wild or genetically modified strains of *S. cerevisiae* can be improved further by the application of the second – physiological – approach. In this case relatively simple control strategies based on a general physiological knowledge of yeast behaviour have been proposed and used for the production of ergosterol.

In fed-batch cultivations according to Shang *et al.* (2006)[22] direct feedback control of ethanol concentration via glucose feeding rate as a manipulated variable with pH control using ammonia was an effective control method for ergosterol production. These authors[23] also studied the effect of different nitrogen sources on ergosterol production and showed that high concentrations of ammonium ions inhibited the accumulation of ergosterol in yeast cells. In the research[24,25] various fed-batch cultivation strategies (constant feed rate, constant glucose concentration, exponential feeding, dissolved oxygen control, exponential fed-batch and dissolved oxygen pulse control) were compared and it was found that a dissolved oxygen pulse control strategy, in which dissolved oxygen was kept at slightly above its critical value of 12 % by glucose pulse feeding, led to the highest ergosterol content.

Shang *et al.* (2008)[26] reported investigations of two control strategies for the simultaneous production of glutathione and ergosterol. The first strategy was based on keeping the ethanol concentration around a constant low level of 1 % while the second one was based on keeping the declining rate of ethanol concentration in the range of -0.1 % to -0.15 % per hour. In both cases the manipulated variable was the glucose feeding rate and an on-off controller was used. The second strategy was much more efficient as far as ergosterol productivity was concerned; however a closer look at the courses of the fermentations indicates that ergosterol content in cells decreased from the beginning to the end in both cases. On-line stabilisation of specific growth rate at a prespecified constant value for improving glutathione production in *S. cerevisiae* was also reported.[27] Besides basic methods used for single loop feedback control of *S. cerevisiae* fermentations, as PID or on-off

controllers, alternative strategies based on intelligent control have been also proposed. For instance, for tracking the set-point of the concentration of ethanol by feed rate of glucose a reinforcement learning algorithm with an adaptive gain was used,[28] or ANN and genetic algorithms were applied for computing an optimal feeding rate profile.[29]

The total yield of ergosterol during the fermentation process is determined by both the ergosterol content in the yeast cells and the amount of yeast biomass. Therefore suitable control strategies should be aimed not only at increasing cell growth but also at maintaining a relatively high or even increasing ergosterol content in the yeast cells.[18] From a downstream processing point of view a third parameter, ergosterol purity, defined as the percentage of ergosterol in the total sterols in the yeast at the end of fermentation, is equally important. In fact purity of the product is the primary indicator of the yeast quality for ergosterol manufacturers.[30] In spite of its great significance the data in the literature on ergosterol purity is really scarce. We can cite only three such publications. Research results of ergosterol biosynthesis in novel melanised fungi were compared with sterol biosynthesis in *S. cerevisiae*.[31] The content of ergosterol as a proportion of total sterols moved from approximately 50 to 61 % whereas the highest content was reached in one strain of *S. cerevisiae*. A report of work on a thermotolerant yeast[17] stated that the original wild type of *S. cerevisiae* investigated showed a content of approximately 80% ergosterol as a proportion of total sterols while one selected thermotolerant strain had 80% fecosterol as a proportion of total sterols with almost no ergosterol. The study on ergosterol production from corn straw hydrolysates[20] only mentioned that the ergosterol obtained had a high purity.

Information on acceptable purity from industry is even more difficult to obtain, because no manufacturer openly reveals this figure. As a reference number we can take analytical results of a final product of a cooperating baker's yeast factory producing yeasts with a high content of ergosterol. Analyses disclosed ergosterol purities in the range 70 – 80 % of total sterols. Apparently a satisfactory level of purity is not always easily attainable.

Many factors both external (temperature, pH, stirrer speed, composition of medium) and

internal (type of fungus, its strain, genetic manipulation, *etc*) must be considered in planning to increase the concentration of ergosterol in yeast biomass, but also how to reduce the amount of contaminating sterols. In our study we therefore focused only on investigating how ergosterol is produced in different physiological states of *S. cerevisiae* during cultivation when all other conditions including those mentioned above were maintained constant. Our aim was to find the cultivation conditions favourable for both a high total yield of ergosterol and for its high purity at the same time.

For our investigation we adopted the definition of physiological states[4] which enabled the estimation of these states from standard on-line reliably measureable process variables[32]. We exposed the yeast culture to different physiological states during fermentation, applying a control strategy based on on-line inference of the current physiological state of the culture. This strategy was implemented in a proprietary knowledge-based control system and was used for enforcing various sequences of physiological (metabolic) states on the culture during experimental cultivations

The results achieved made it possible to formulate suggestions for the fed-batch production of ergosterol capable of obtaining a high concentration of yeast in the cultivation medium, a high concentration of ergosterol in the biomass (ergosterol content) and a low content of other contaminating sterols (high ergosterol purity) at the end of the fermentation, all at the same time. Taking into account the ergosterol purity as an equivalent parameter can be seen as one of the novelties of this study.

2 Materials and methods

2.1 Physiological modelling and control

The strategy used for the control of the fed-batch experiments was based on physiological state control.[32] On the basis of the knowledge of the on-line inferred current physiological state and the desired physiological state the strategy was able to recommend the current and future set points of the CO_2 concentration in the exit gas for a PID controller during the cultivation in order to maintain

the culture within the desired physiological state or to recommend directly a future course of the flow rate of the medium as a manipulated variable to force the culture into the desired physiological state from a momentarily undesirable one. Because the inferential procedure is based on and works primarily with values of *RQ* and *EtOH* variables, the CO_2 concentration in the exit gas was chosen as the controlled variable. Selection of CO_2 concentration for control purposes thus allowed us to reserve the variables *RQ* and *EtOH* entirely for indication of metabolic states, as these were not directly controlled. Another reason why we preferred the CO_2 concentration as the controlled variable was that according to control theory the CO_2 concentration is an output variable (produced by cells). In the physiological control system the CO_2 concentration was not a target controlled variable but only an auxiliary one, because in this case the target "variable" was the whole metabolic state, which is characterized by the values of several process variables, see Fig. 1 Structure of measurement and control system. This approach enabled us to realize various pre-specified sequences of physiological states of the culture during cultivation.

Each physiological state of the cultivated microorganism *S. cerevisiae* is characterised by the effects of a group of metabolic pathways, which are activated and used by the microorganism depending upon current extracellular and intracellular conditions.[4] Three different metabolic pathways exist in *S. cerevisiae* yeasts for growth on glucose and/or ethanol: (1) complete oxidation of sugar to CO_2 and H_2O, (2) partial fermentation of sugar to ethanol and CO_2, and (3) complete oxidation of ethanol to CO_2 and H_2O. Which of the metabolic routes is followed depends on the availability and relative amounts of sugar, and ethanol with respect to the respiratory capacity of the cells, when oxygen is maintained in growth nonlimiting surplus.[4]

Six main "metabolic states" can be distinguished, in which the culture can remain for long periods of time within the cultivation[33] characterised by different productivity and yield coefficients:

(MS1) oxidative growth on glucose only (*without production of ethanol, the concentration of sugar is less than a critical level*),

(Border State 2) a "border" state between states MS1 (oxidative growth on glucose

only) and MS3 (oxidative-fermentative growth on glucose) formed by the narrow sections of these states adjacent to their boundary line (*concentration of ethanol remains constant*),

(MS3) oxidative-fermentative growth on glucose, aerobic fermentation (*with production of ethanol caused by the surplus of sugar*),

(MS4) oxidative growth on glucose and ethanol (*ethanol and sugar are simultaneously utilised when the concentration of sugar is less than a critical level*),

(MS5) oxidative growth on ethanol only (*ethanol is consumed when sugar is absent in the medium*),

(MS6) starvation (*glucose and ethanol are not available in the medium and feed rate is zero*).

The "border" state 2 is defined rather artificially, because it is suitable for maintaining culture growth at a critical specific growth rate, which gives optimal productivity and yield of biomass and it is relatively easy to achieve. Nevertheless our studies described below forced us to split the original Border State 2 into two separate states - Oxidative Border State and Oxidative-Fermentative Border State - and consider them individually. These states are denoted as MS0 and MS2 in the text.

For the application of the physiological control strategy, a knowledge-based control system named BIOGENES© was used.[34,35] BIOGENES© is a rule-based system with a knowledge base comprising more than 250 rules mainly for tasks of process state classification and supervisory control. For identification of the current metabolic state it uses a set of easily measurable and computable variables that explicitly reflect cell metabolism: ethanol concentration – *EtOH*, ethanol concentration trend – d*EtOH*/d*t*, dissolved oxygen tension – *DOT*, CO_2 concentration in exit gas – CO_2, respiratory quotient – *RQ*, and glucose feed rate – *Fm*.

The inference engine ran with a period of 1 minute and used symbolic rather than numerical values of these variables. Because all measured variables were quite sensitive to noise, prior to their transformation into the symbolic form they were filtered via a Butterworth second order filter.

Genuine identification of metabolic states consisted of two consecutive steps. In the first step, all six metabolic states were rated by a classification algorithm based upon the last five symbolic values of the respiration quotient *RQ*, ethanol concentration *EtOH*, ethanol concentration trend d*EtOH*/d*t*, and substrate feed rate F_m. The rating scheme used is given in Table 1. For example, when the combination of values for one minute (from the window of the last four minutes) for given variables meets the conditions stated in their supporting evidences column, the pertinent metabolic state receives one plus point, and so on for another minute. In the second step all metabolic state ratings awarded were subjected to correction by auxiliary situation-specific rules (based on CO_2 and *DO* values) if applicable. The system could use an extra set of rules for solving conflicting situations where more than one metabolic state shared the highest rating. Subsequently, the metabolic state with the highest rating was selected as the currently dominant state. The inference algorithm of the BIOGENES© system was robust enough and performed well even in the case when only one of the *EtOH* or *RQ* measurements was available which was the case in our last experiment.

In its supervisory control module the knowledge-based control system uses inferred and desired metabolic states together with other inferred indicators, such as for example substrate feeding quality for determination of the CO_2 set point or *Fm* adjustments. The whole algorithm is rather elaborate and was fully explained in the original article.[34]

2.2 Microorganism and cultivation medium

Saccharomyces cerevisiae yeast, strain D7, prepared as an UV mutant giving improved ergosterol biosynthesis, was provided by a yeast factory.

An optimised semi-synthetic cultivation medium consisting of glucose (125 or 250 g L^{-1}), yeast extract (DIFCO) (31.2 g L^{-1}), ammonium sulphate (7.8 g L^{-1}), potassium dihydrogen phosphate (3.7 g L^{-1}), magnesium sulphate (3.1 g L^{-1}), calcium chloride (1.25 g L^{-1}) in mains water, was used.

2.3 Chemical assays

Biomass determination – 5 ml samples were centrifuged, the sediment washed in distilled water, spun down again, and gravimetrically determined after drying at 105 °C.

Sterols (ergosterol) – ergosterol and three other sterols, which were considered to be contaminants of the sterol fraction in the yeast biomass, were analysed using an HPLC reverse-phase column, type C18 (250×4 mm). Prior to HPLC determination a 3 hour alkaline hydrolysis of cells (3 ml of suspended cells + 3 g KOH) at 100 °C was performed and followed by extraction in diethylether, evaporation of the ether, and final dissolution in a mobile phase (methanol-water in the ratio 95:5). Evaluation of peak areas was carried out by a standard method. By comparing peak areas of samples against the peak area of an ergosterol standard a value of the total content of sterols in the yeast dry biomass was calculated. The purity/impurity of the ergosterol fraction was calculated from the total sterol and ergosterol contents in the biomass. The contaminating sterols were precursors Ergosta-5,7,24(28)-trienol and Dehydroergosterol (chemically Ergosta-5,7,22,24(28)-tetraenol) and side product Dihydroergosterol (chemically Ergosta-5,7-dien-3βol). The chemical structure of the analyzed and detected sterols were not determined in our laboratory, but the HPLC analyses were done in our laboratory.

Glucose and ethanol – these components were determined in the supernatant by HPLC using a column filled with OSTION 0800 in H^+ cycle. The mobile phase was 0.005 mol L^{-1} sulphuric acid.[36]

2.4 Bioreactor, monitoring and control

Experiments were carried out in a New MBR (Switzerland) laboratory bioreactor with a working volume of 7 L, which was equipped with an analogue control unit IMCS 2000 (PCS Switzerland) and a PC for control purposes. The analogue control unit was used to measure and stabilize temperature and pH of the medium, stirrer speed, air flow, and foam level. Dissolved oxygen tension was measured by a polarographic oxygen probe (Mettler Toledo). For supplying feeding medium to the bioreactor, a DP 200 peristaltic pump (New Brunswick Scientific) operating in doses was used. SERVOMEX Type 1100A and 1400B analysers provided measurement of the oxygen and

carbon dioxide concentrations, respectively, in the exit gas. The ethanol concentration in the outlet gas was measured continuously by a METREX instrument (*constructed by ICT Prague*).

The proprietary knowledge-based control system BIOGENES© (*developed by ICT Prague, Dept. of Computing and Control Engineering*) running on a PC performed all real-time monitoring and control functions. This system consisted of two levels. The basic control level fulfilled all standard process control tasks, the upper knowledge-based supervisory level executed more intelligent tasks of classification and intelligent advising. The basic control level was written using the GENESIS® for Windows 3.5 package (Iconics, USA). The knowledge-based supervisory level was written using the expert system shell CLIPS® 6.05 (NASA's Johnson Space Center), and was interconnected with the GENESIS® for Windows level.[34,35]

3 Results and discussion

3.1 Description of experiments

A series of 11 experiments was carried out. In all cultivations samples were taken every hour for the determination of biomass, glucose and ethanol concentrations in the medium, and for the analyses of sterols including ergosterol in the biomass. From the values of biomass concentration the specific growth rate μ was calculated every hour. The control system continually monitored the dissolved oxygen tension, *DOT*, the concentrations of CO_2, O_2 and *EtOH* in the exit gas, and the medium feed rate, *Fm*, and logged the values of these every half a minute. The respiratory quotient, *RQ*, was further calculated from the concentrations of CO_2 and O_2 in the exit gas. The values of *EtOH*, *RQ*, *Fm* and also *DOT* and CO_2, which comprehensively characterize the metabolic state, served as data for its continual inference by the knowledge-based control system (see section *2.1*). The sequences of the prescribed metabolic states and actually realised metabolic states inferred during the experiments are outlined in Table 2.

Evaluation of the time-courses of the purity of ergosterol (erg_{pur}) indicated that the culture behaved differently in different experiments when enforced into Border State 2. In Experiment 5 (depicted here in Fig. 2) the purity of ergosterol sharply declined – as it did throughout the cultivation period - while in Experiment 6 the purity remained almost constant from the beginning to the end of cultivation. A more detailed study revealed the reason. In Experiment 5 the culture was maintained just on the pure oxidative growth side of Border State 2 while in Experiment 6 the culture was maintained on the oxidative-fermentative side of Border State 2.

After these findings we split Border State 2 into two individual states, named the Oxidative Border State and the Oxidative-Fermentative Border State and denoted them as MS0 and MS2, respectively. Such notation enabled us to maintain the continuity of the notation of the other metabolic states. The final version of the metabolic states investigated is listed in Table 3.

The overall results obtained from all the experiments are summarised in Table 4, in which the total efficiency of the process of biomass production was defined as the product of the yield coefficient, $Y_{X/S}$, and the biomass volumetric productivity, p; the total efficiency of the process of ergosterol production was defined as the product of the ergosterol yield coefficient and the ergosterol volumetric productivity, $Y_{erg/S} \cdot p_{erg}$.

At this stage, it must be pointed out that in all experiments except the last one (No. 11) the primary aim was not to maintain optimal conditions for ergosterol production during the whole cultivation but to expose the culture to different sequences of induced metabolic states for studying the production of ergosterol under different conditions.

The experiments carried out may be separated into several groups depending on the behaviour of the culture during cultivation and the final results achieved. Cultivations Nos. 1, 2, 3 and 4 were controlled in order to examine the behaviour of the culture with overfeeding at the beginning of the fermentation and characterized also by high specific growth rates. The metabolic states of these cultures remained for most of the time in metabolic state MS3 and only marginally in MS4 or MS5. In the cultivations with the two highest initial specific growth rates (Experiments 1 and 2; see Table 4), a

considerable increase in ethanol concentration in the medium was observed owing to overfeeding of glucose. Thus, substrate was not used efficiently for biomass production, which was reflected in the overall yield of biomass $Y_{x/s}$ being the lowest (0.11 – 0.22 g g^{-1}). Likewise, the levels of the total efficiency of ergosterol production, $Y_{erg/S} \cdot p_{erg}$, and final ergosterol concentration, erg_f, for these runs were also the lowest of all the experiments. However, the purity of ergosterol obtained was 86 – 93 %, the highest of all of the experiments. The purity erg_{pur} increased, remained the same or decreased slightly (with an average difference of -0.8% from the beginning to the end of fermentations). Experiment 1, belonging to this group, is shown in Fig. 3 as an illustration. In the figure, the first graph shows plots of the variables that characterize the metabolic states of the culture, while the second graph depicts the time-courses of the variables that relate to biomass and ergosterol together with the actual sequence of metabolic states enforced during the experiment.

The second group of experiments comprising cultivations 5, 6, and 7 (illustrated, for Experiment 5, in Fig. 2) was characterized by a moderate final total efficiency of ergosterol production, $Y_{erg/S} \cdot p_{erg}$, a moderate final ergosterol concentration erg_f and the lowest final biomass concentration X_f among the cultivations. The time-courses of these fermentations could be regarded as a standard baker's yeast fed-batch production profile with slight variations, as described in Table 2.

The final purity of ergosterol obtained in these cultivations was in the lower range 75 – 79 % and dropped on average by 17.8 % during the fermentations. Experiment 5 was the first instance of a fall in ergosterol purity during Oxidative Border State MS0, in fact MS0 oscillating with MS1.

On the basis of the findings of Experiment 5 two experiments 8 and 9 were proposed to verify the hypothesis that the Oxidative Border State MS0 is not favourable to erg_{pur}, as shown by its decline during this state. Both experiments were carried out with long phases of Oxidative Border State MS0 lasting 13.5 hours and 21.5 hours, respectively. These cultivations confirmed the hypothesis, as it can be seen in Fig. 4, where Experiment 8 is represented. In both experiments there was a very similar sharp drop in erg_{pur} from 95 % to 65 – 67 % during State MS0. Oxidative Border

State MS0, which lasted for nearly all of Experiment 9, moreover had a deleterious effect on the ergosterol content of the cells, *Erg*, because this experiment produced the lowest ergosterol productivity and was the only one in which the final *Erg* content decreased (see Table 4).

The remaining two cultivations, 10 and 11, belong to the last group of experiments in terms of their comparable results and the partial similarity of their sequences of induced metabolic states, even if they were designed with different aims. The first one was designed to test the capability of the knowledge-based system itself by exposing the culture to a sequence of the greatest number of metabolic states. The second one was designed in the light of the earlier findings as a possible new cultivation control strategy, with the exclusion of metabolic state MS0 and its adjacent states MS1 and MS2, along with MS5 (see below) and therefore involving metabolic states MS3 and MS4 only.

The actual sequences of the metabolic states achieved in these cultivations were {1,0,3,4,0,3,4} and {4,3,1,3,4,3,4}, respectively. The results from the two experiments are shown graphically in Fig. 5 and Fig. 6. Because ethanol concentration *EtOH* was not measured during Experiment 11 due to failure of the analyser, this variable is not shown in Fig. 6. As it can be seen from Table 4 both cultivations achieved the best values of biomass and ergosterol productivities and biomass and ergosterol total efficiencies and also the best values of biomass and ergosterol final concentrations of all the cultivations. However, the two cultivations also showed differences. Even if the biomass results were comparable, cultivation 11, controlled according to the new cultivation strategy, produced approximately 50 % better results as far as productivity, efficiency and final concentration of ergosterol are concerned. An increase in biomass ergosterol content by 1.4 % during the cultivation was also the best achieved.

The evolution of the purity of ergosterol confirmed the foregoing results. While during cultivation 10 erg_{pur} declined during both occurrences of metabolic state MS0 up to its final value 80 %, in cultivation 11, erg_{pur} remained nearly constant with a final value of 86 %. Both final purities lay within the second best quartile of the purities achieved (80-89%) and are sufficient for subsequent down-stream processing.

3.2 Changes in sterols composition

To quantify the changes in erg_{pur} within individual metabolic states, all the records of variables within all the experiments, which were sampled every 30 seconds, were classified as belonging to one of the metabolic states listed in Table 3. In all, 32051 records were made within 267 hours of all experiments. The number of records classified as state MS6 (starvation) was only 183, which is not statistically significant. Then the changes of erg_{pur} in % h^{-1} were calculated for every hour of cultivation as a forward difference of erg_{pur} values. These differences were at first pre-processed by monotonising the data of their time-course with the removal of outliers. The resulting differences were then assigned to the individual records and by means of these records to the individual metabolic states. It enabled us to calculate the prevailing trends in changes of erg_{pur} within individual metabolic states for all 11 experiments together. The trends were expressed as a percentage of time of each state in which erg_{pur} was decreasing, was increasing, or was constant. For the classification of changes of erg_{pur} two limit values were used {-0.1, 0.1} % h^{-1}. If the change in the record was between these values, the relevant record was included in the set with constant erg_{pur} and marked STEADY, in other cases a record was marked UP or DOWN. These chosen limit values enabled us to classify only minimal changes of erg_{pur} as STEADY. Table 5 contains the results together with the row marked TOTAL, which contains the numbers of records belonging to the individual metabolic states, and so provides information on the percentile occurrence of these states within all the cultivations. For instance state MS0 was encountered in 5965 records measured every half minute during all 11 experiments and in 92% of them the purity of ergosterol was declining, i.e. in 5488 records.

The results summarized in the first part of Table 5 confirm that erg_{pur} declined almost all the time during Oxidative Border State MS0. Surprisingly in the adjacent metabolic states MS1 and MS2

an increase in erg_{pur} was never seen. The only three metabolic states in which growth of erg_{pur} occurred were MS3, MS4 and MS5. The purity of ergosterol remained steady or increased during half of MS3, MS4 and MS5. From the point of view of an optimal production of ergosterol the cultivation should avoid metabolic states MS0, MS1 and MS2, in order not to reduce erg_{pur}.

3.3 Changes in ergosterol concentration

Changes in the concentration of ergosterol in the cells (the content of ergosterol in the dry weight of biomass *Erg*) in relation to metabolic states were also studied. The percentages of time within each metabolic state when the content of ergosterol increased, decreased or remained constant were calculated as before and are summarised in the second part of Table 5. The content of ergosterol *Erg* increased during two thirds of the records of metabolic states MS1 and MS2 and 60% of those of MS3 whereas it increased during one half of the records of the remaining statistically significant states MS0, MS4 and MS5. The worst case for ergosterol production was state MS5, in which *Erg* decreased for more than one third of its operating period. The highest rate of *Erg* increase was 0.27 % h^{-1} and was achieved in several cultivations, for example in Experiment 11 from the 2nd to the 6th cultivation hour.

3.4 Changes in biomass concentration

Because the total yield of ergosterol is determined not only from the ergosterol content in the cells, but from the amount of yeast biomass produced at the same time, it is necessary to evaluate the results of the experiments also from the perspective of biomass concentration. The conditions for optimal production of *S. cerevisiae* are theoretically known and they are met when cells are growing at a critical specific growth rate. In this state the available respiratory capacity of the cells is fully exploited for oxidative growth and no fermentative growth on glucose appears. It corresponds to Border State 2 from the definitions of metabolic states.

In the description of our experiments the growth of biomass is characterised by the courses of

specific growth rate μ. When we follow these courses of μ during a particular experiment we can see very different values of μ in the same metabolic state. There were remarkable differences for example in two occurrences of MS0 in Experiment 10 and in three occurrences of MS3 in Experiment 11. This downwards trend in μ was caused by different growth phases (exponential, stationary) through which the culture moved during cultivation. For this reason, it was not possible to carry out the same pattern of analysis for biomass production as in previous sections. Nevertheless the overall results of experiments with different prevailing metabolic states (for example MS0 in Exp. 8 versus MS3 in Exp. 11) confirm that for biomass production state MS3 is the better one. Theoretically the optimal state is also MS2, because in this state as in MS3 there is the guarantee of complete exploitation of the respiratory capacity of the cells.

3.5 Optimal control of production cultivations

When considering the optimization of the overall process of ergosterol production, we are confronted by a number of conflicting requirements. We are demanding the highest yield, maximal biomass growth and maximal ergosterol production productivity. At the same time the cells should contain the purest possible ergosterol at the end of the cultivation. Data in Table 5 enabled us to characterize the contribution of individual metabolic states to the effective production of ergosterol defined above. So in metabolic state MS0 erg_{pur} almost steadily declines. Therefore it is necessary to avoid this state during cultivation. In metabolic states MS1 and MS2 erg_{pur} did not increase, but on the other hand under these two states *Erg* grew the most. Metabolic state MS3 was characterized by an increasing or steady erg_{pur} and an increasing *Erg* much like that in MS1 and MS2. This suggests that MS3 is a good candidate for including in an optimal cultivation. Ergosterol purity erg_{pur} behaved in the same way under MS4 as under MS3 or MS5, in that it increased or was steady in half of the instances, while *Erg* grew, albeit more slowly than in MS1, MS2 or MS3, but with the smallest percentage of possible decrease. Even if erg_{pur} grew the most under MS5, this state was characterized

by the greatest decrease of *Erg* – approximately over one third of its instances – and so it is not considered to be favourable.

In the light of these findings, a new cultivation strategy for the ergosterol production process satisfying the above-mentioned criteria can be defined as follows:

(1) Metabolic state MS0 should be strictly avoided during the cultivation.

(2) From the ergosterol purity point of view it is better also to avoid states MS1 and MS2.

(3) The culture should not be kept in metabolic state MS5 because of the greatest possibility of declining ergosterol content during this state.

(4) After a short initial adaptation phase, the culture should be maintained in metabolic state MS3, i.e. in oxidative-fermentative growth on glucose. This state is favourable for the greatest increase in ergosterol content, for the greatest probability of increasing or maintaining the purity of ergosterol, and also from the point of view of production of biomass. It is necessary to emphasize that during MS3 the value of respiration quotient *RQ* must be maintained below 1.3 to avoid overfeeding leading to massive production of ethanol. Because of all these conditions a slow production of ethanol will take place. Therefore it will be desirable to include a short final phase in which ethanol is fully consumed at the end of the cultivation.

(5) In the final phase metabolic state MS4, characterized by oxidative growth on glucose and ethanol concurrently, should therefore be maintained.

Following these rules a new cultivation strategy was designed and carried out in Experiment 11. This strategy involved imposing two pairs of MS3 + MS4 metabolic states. For the sequence of metabolic states achieved in practice, see Fig. 6. In this cultivation the control strategy applied achieved the desired results, which may be considered as having fulfilled all the above-mentioned criteria: productivity, efficiency and final concentration of ergosterol and last but not least ergosterol purity. When we compared the average results from the second group of experiments, which can be regarded as standard baker's yeast fed-batch cultivations, with the results of this new

control strategy we obtained the following figures: the total efficiency of ergosterol production $Y_{erg/S}{\cdot}p_{erg}$ 32.14x10^{-6} and 103.84x10^{-6} g L^{-1} h^{-1}, the final content of ergosterol in dry biomass Erg_f 1.91 % and 2.43 %, and the ergosterol final purity erg_{pur} 77 % and 86 %, respectively. The ergosterol purity achieved (86%) was better than the purity reported from industry (70-80%). In comparison with figures for the final content of ergosterol in the dry biomass mentioned in the literature for fed-batch cultivations of wild *S. cerevisiae* strains (2,35 %,[20] 1.25 %[22] and 2.1 %,[23]) and of genetically modified strains (1 %,[15] 1.37 %,[26]) the value 2.43 % achieved by the new strategy was very good. The sequence of metabolic states MS3 and MS4 and their precise timing can be optimized even further. Considering the important aspect of high biomass production it would be advantageous to maintain state MS3 as long as possible and to switch to state MS4 at the precise moment that ensures complete concurrent depletion of sugar substrate and ethanol at the end of the cultivation.

4 Conclusions

Investigations of variations in ergosterol concentration and its purity under different conditions of physiological state of the culture of *Saccharomyces cerevisiae* yeast have enabled us to identify conditions under which substantial increases in ergosterol concentration in the biomass and ergosterol purity as a proportion of total sterols can be achieved simultaneously. Using the new knowledge it has been possible to define several general rules which should be taken into account in the design of any cultivation strategy for efficient ergosterol production. The results achieved have considerable significance also for downstream processing of yeasts and production of pure ergosterol and vitamin D_2, and are therefore relevant to improving the performance of large scale production processes.

The proposed physiological control strategy could be executed either by using a knowledge-based control system, as reported here, or by any standard physiological control strategy based on and working with easily measurable and derivable variables such as *EtOH* and *RQ*. The physiological

approach implemented is robust enough, in that a strategy providing physiological control is able to react rapidly and flexibly to unforeseen changes that are taking place during the cultivation. In our opinion the concept of metabolic states adopted here has potential to combine metabolic regulation with bioprocess control.

Nomenclature

CO_2 = Content of carbon dioxide in exit gas (% v/v)

CO_2sp = Set point of CO_2 (% v/v)

dcw = Concentration of yeast dry biomass (g L^{-1})

DOT = Dissolved oxygen tension (% saturation)

d*EtOH*/d*t* = EtOH trend (% v/v h^{-1})

EtOH = Concentration of ethanol in exit gas (% v/v)

erg = Concentration of ergosterol (g L^{-1})

erg_f = Final concentration of ergosterol (g L^{-1})

erg_{pur} = Percentage of ergosterol in total sterols: purity (% w/w)

Erg = Ergosterol in the dry weight of biomass (% w/w)

Erg_0 = Initial ergosterol in the dry weight of biomass (% w/w)

Erg_f = Final ergosterol in the dry weight of biomass (% w/w)

Erg_m = Maximum ergosterol in the dry weight of biomass (% w/w)

Fm = Medium feed rate (L h^{-1})

MS = Metabolic state

MSdesir = Desired metabolic state

MSestim = Estimated metabolic state

O_2 = Content of oxygen in exit gas (% v/v)

p = Biomass volumetric productivity (g L^{-1} h^{-1})

p_{erg} = Ergosterol volumetric productivity (g L^{-1} h^{-1})

RQ = Respiratory quotient (CO_2 produced/ O_2 consumed) (mole $mole^{-1}$)

t = Time (h)

X = Concentration of yeast dry biomass (g L^{-1})

X_f = Final concentration of yeast dry biomass (g L^{-1})

$Y_{erg/S}$ = Ergosterol yield coefficient (g g^{-1})

$Y_{erg/S} \cdot p_{erg}$ = Total efficiency of ergosterol production (g L^{-1} h^{-1})

$Y_{X/S}$ = Biomass yield coefficient (g g^{-1})

$Y_{X/S} \cdot p$ = Total efficiency of biomass production (g L^{-1} h^{-1})

μ = Specific growth rate (h^{-1})

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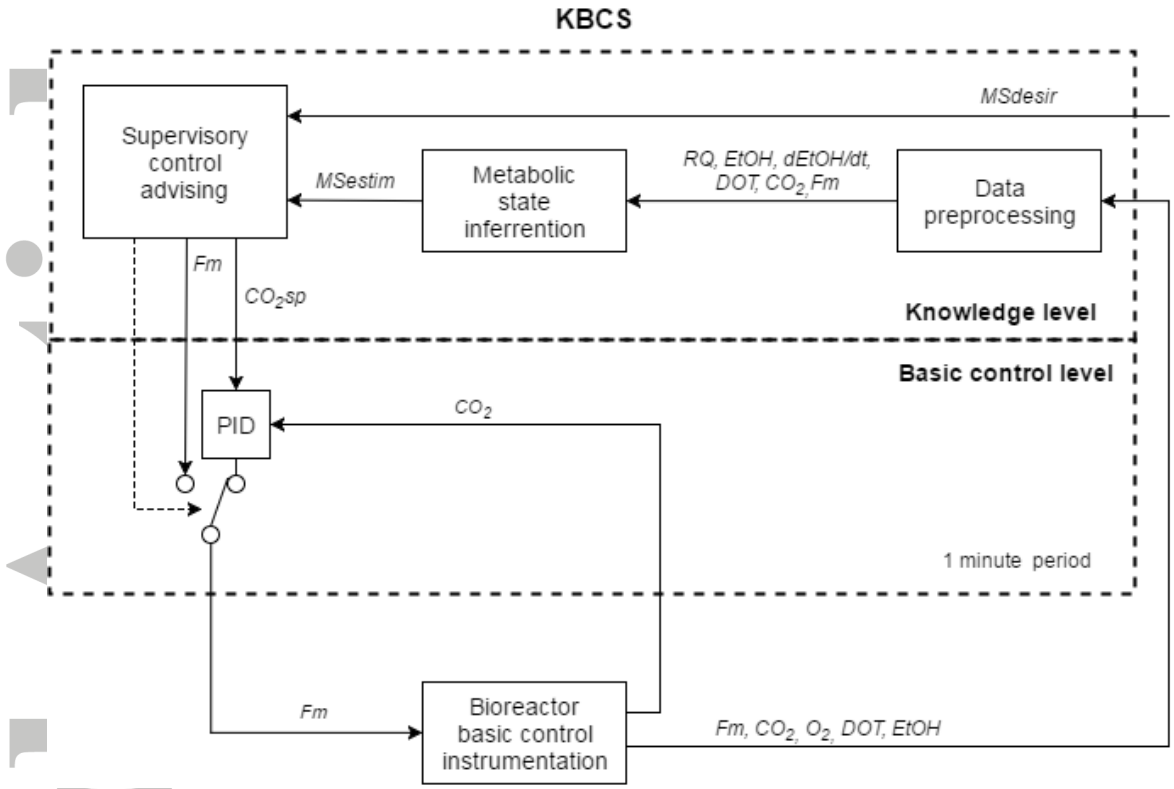

Fig. 1 Structure of measurement and control system

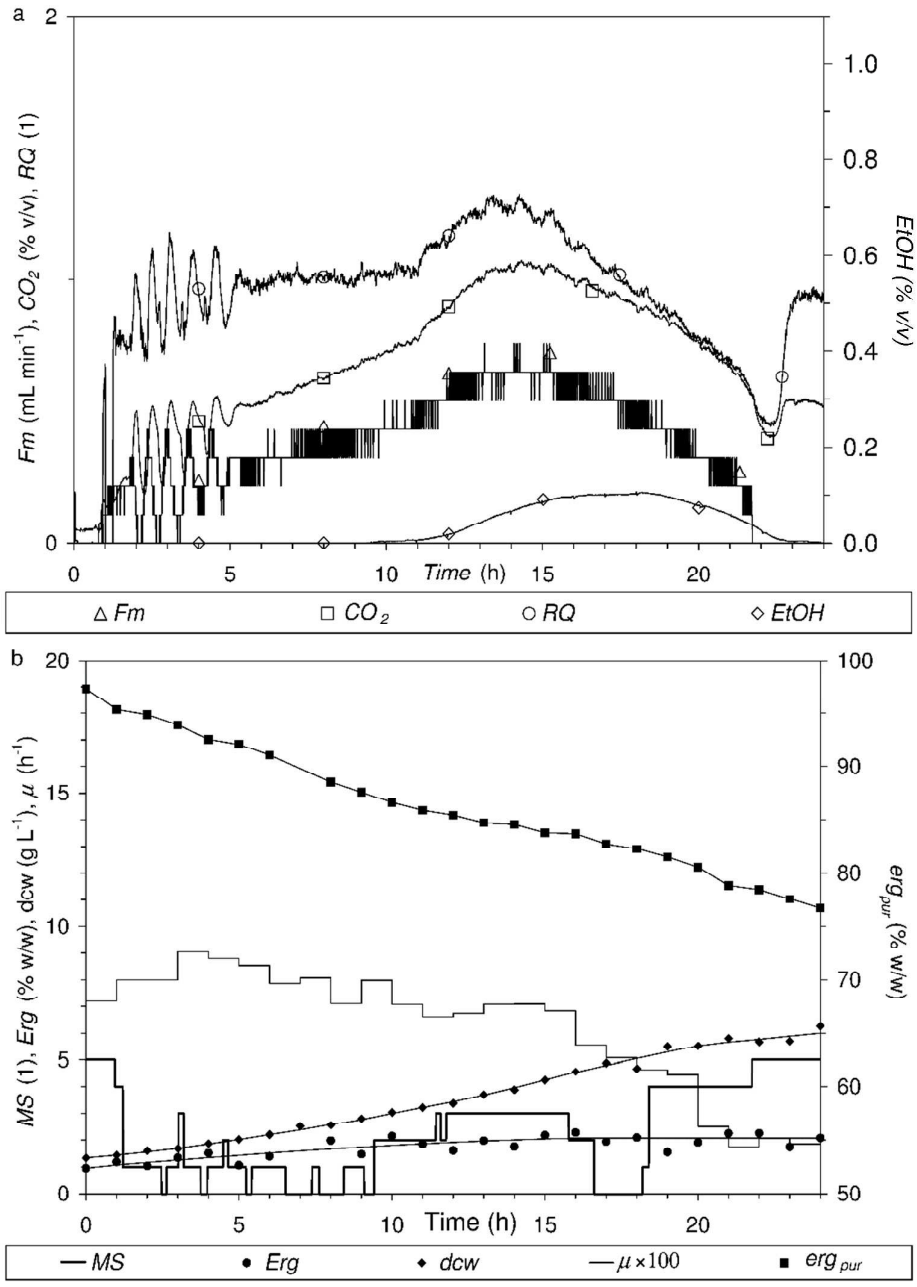

Fig. 2 Experiment 5 – The experiment carried out as a standard Baker's yeast productive fermentation – (a) time-course of quantities important for the classification of metabolic state: medium feed rate *Fm*, ethanol concentration *EtOH*, exit gas carbon dioxide content CO_2 and respiratory quotient *RQ*, (b) time-course of metabolic state *MS* and determined values of dry cell weight *dcw*, specific growth rate μ, ergosterol content *Erg,* ergosterol purity erg_{pur} as data points with processed values as lines

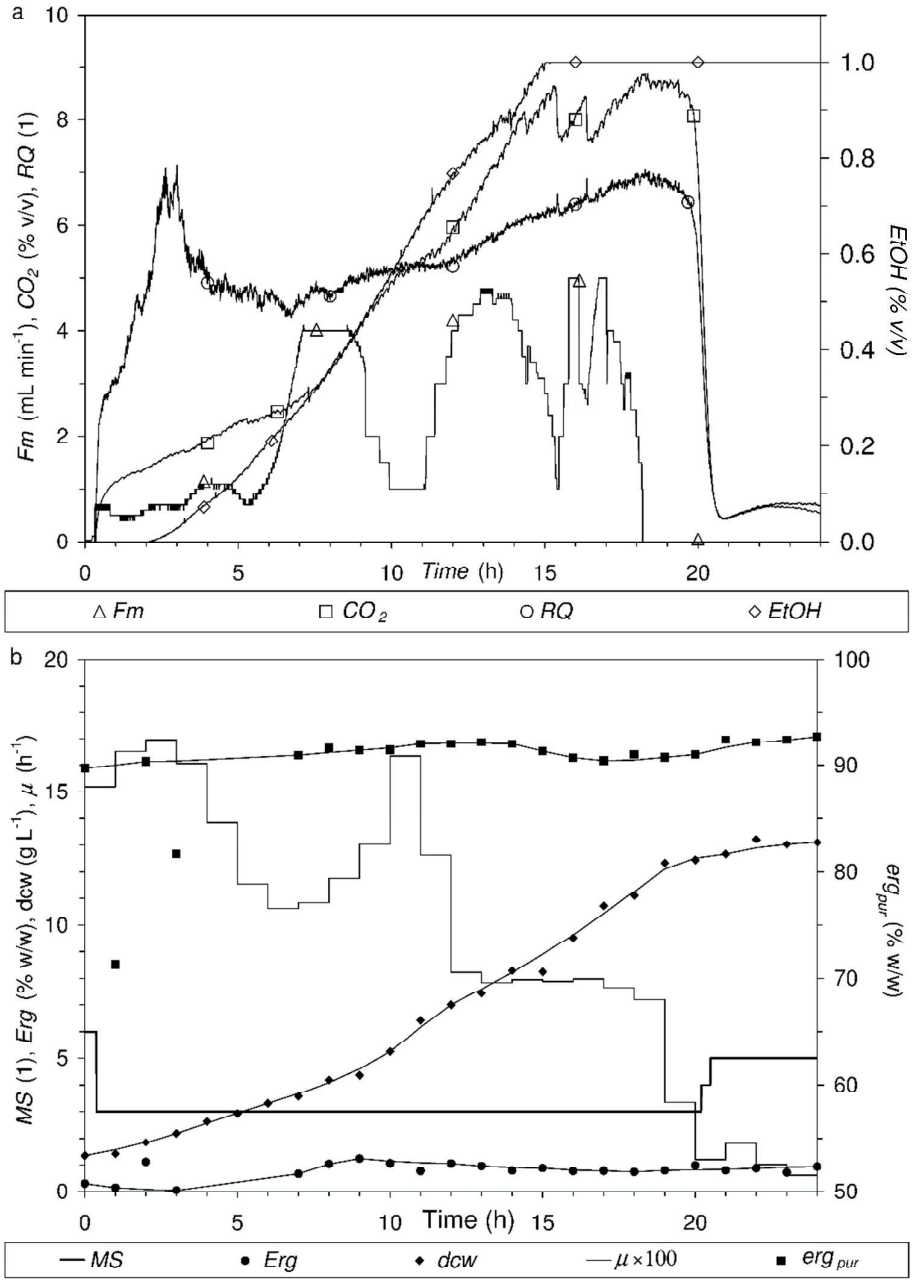

Fig. 3 Experiment 1 – The experimental aim was to study the response of the culture when heavy overfeeding and consequently high specific growth rate occurs during cultivation – (a) time-course of quantities important for the classification of metabolic state: medium feed rate *Fm*, ethanol concentration *EtOH*, exit gas carbon dioxide content CO_2 and respiratory quotient *RQ*, (b) time-course of metabolic state *MS* and determined values of dry cell weight *dcw*, specific growth rate μ, ergosterol content *Erg,* ergosterol purity erg_{pur} as data points with processed values as lines

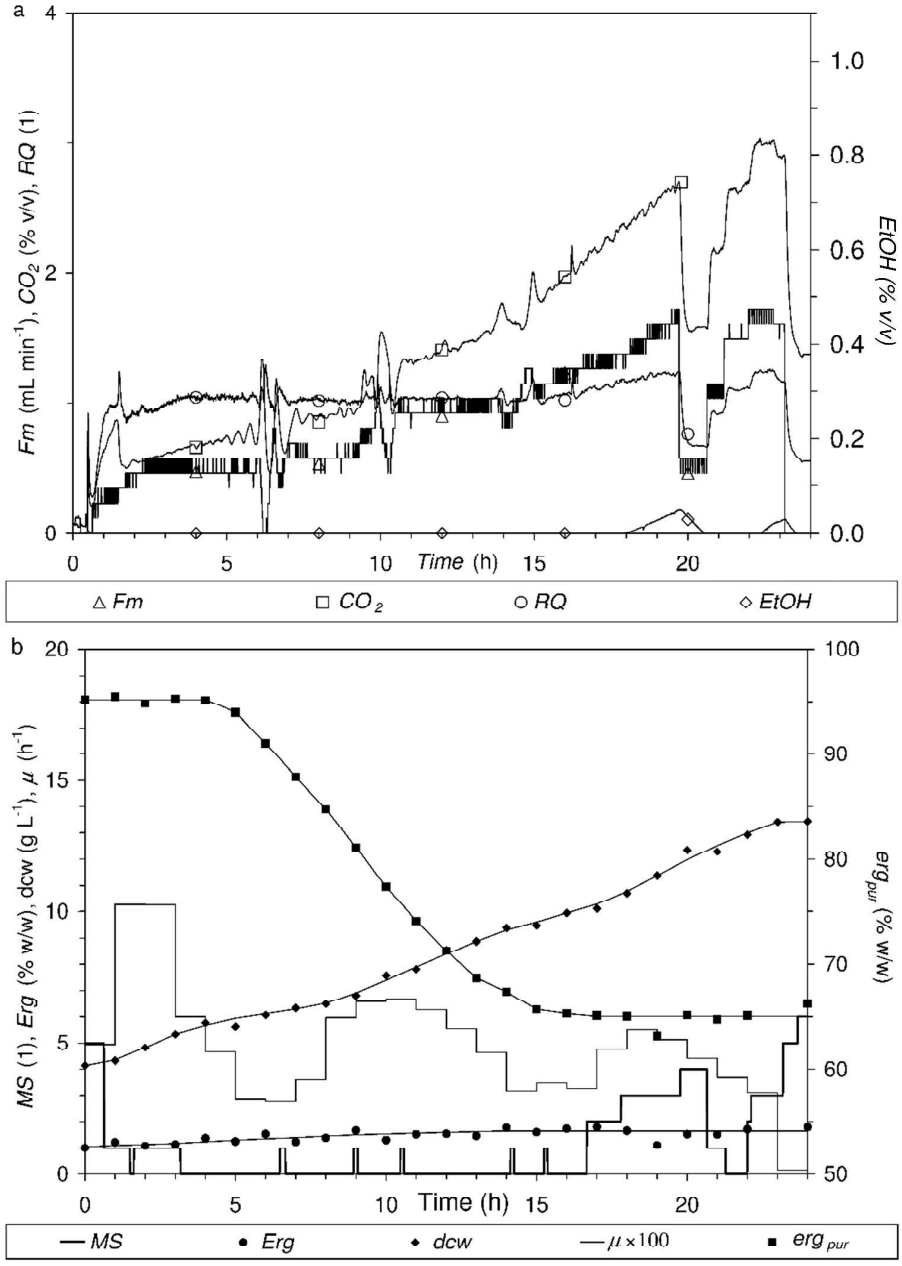

Fig. 4 Experiment 8 – A predetermined chronological sequence of metabolic states {1,0,3,1,0,3} was examined – (a) time-course of quantities important for the classification of metabolic state: medium feed rate *Fm*, ethanol concentration *EtOH*, exit gas carbon dioxide content CO_2 and respiratory quotient *RQ*, (b) time-course of metabolic state *MS* and determined values of dry cell weight *dcw*, specific growth rate μ, ergosterol content *Erg,* ergosterol purity erg_{pur} as data points with processed values as lines

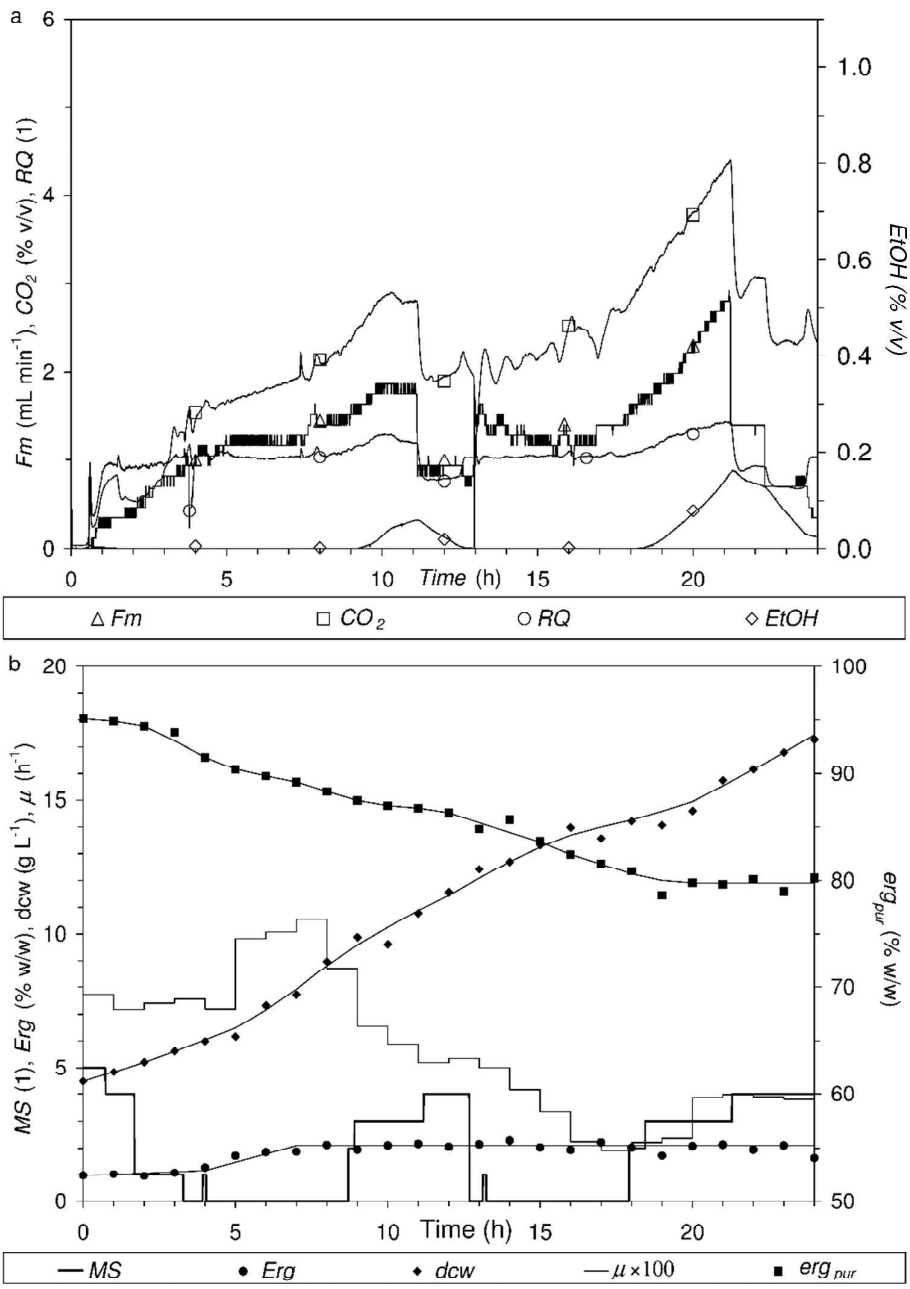

Fig. 5 Experiment 10 – The experiment aimed to test the knowledge-based system. Therefore the predetermined chronological sequence of metabolic states {1,0,3,4,0,3,4} was enforced and examined – (a) time-course of quantities important for the classification of metabolic state: medium feed rate *Fm*, ethanol concentration *EtOH*, exit gas carbon dioxide content CO_2 and respiratory quotient *RQ*, (b) time-course of metabolic state *MS* and determined values of dry cell weight *dcw*, specific growth rate μ, ergosterol content *Erg,* ergosterol purity erg_{pur} as data points with processed values as lines

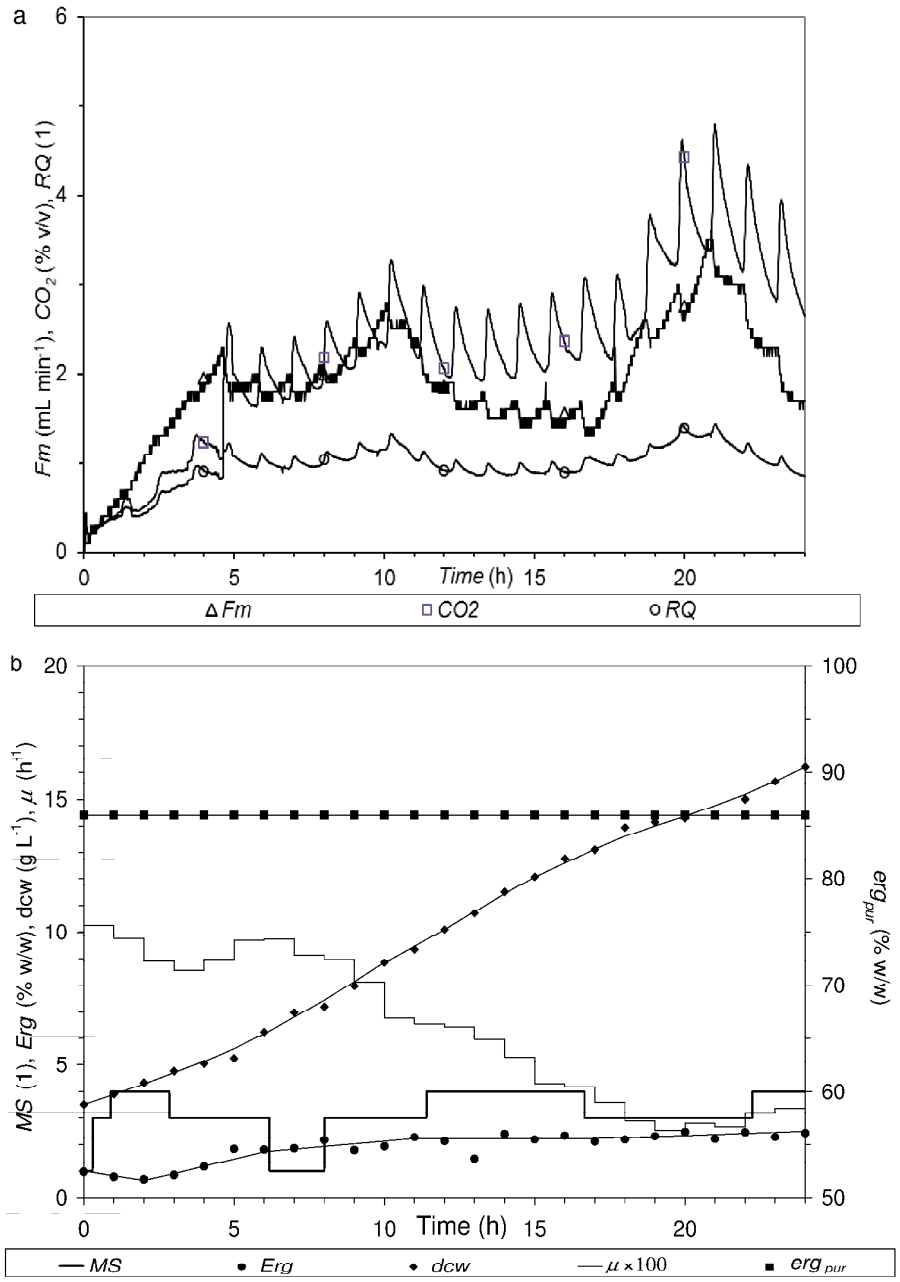

Fig. 6 Experiment 11 – The sequence of metabolic states MS = {3,4,3,4} was chosen and enforced as a new control strategy – (a) time-course of quantities important for the classification of metabolic state: medium feed rate *Fm*, exit gas carbon dioxide content CO_2 and respiratory quotient *RQ*, (ethanol concentration *EtOH* was not measured) (b) time-course of metabolic state *MS* and determined values of dry cell weight *dcw*, specific growth rate μ, ergosterol content *Erg,* ergosterol purity erg_{pur} as data points with processed values as lines

Table 1

Rating scheme used for metabolic state identification in BIOGENES control system[34]

	Supporting evidences (+ points awarded)			**Contradicting evidences** (− points awarded)		
State	***EtOH* and d*EtOH*/d*t***	***RQ***	F_m	***EtOH***	***RQ***	F_m
MS1	*EtOH*≈0	*RQ*≈1	F_m>0	*EtOH*>0	*RQ*≫1	F_m=0
MS2	*EtOH*>0 + d*EtOH*/d*t*≈0	*RQ*≈1.1	F_m>0		*RQ*<1	F_m=0
MS3	*EtOH*>0 + d*EtOH*/d*t*>0	*RQ*≫1	F_m>0		*RQ*<1	F_m=0
MS4	*EtOH*>0 + d*EtOH*/d*t*<0	*RQ*≈0.8	F_m>0	*EtOH*≈0	*RQ*≈1.1 or *RQ*≫1	F_m=0
MS5	*EtOH*>0 + d*EtOH*/d*t*<0	*RQ*≈0.6	F_m=0	*EtOH*≈0	*RQ*≈1.1 or *RQ*≫1	F_m>0
MS6	*EtOH*≈0	*RQ*≈0	F_m=0	*EtOH*>0		F_m>0

Table 2

Experiments and control strategies used

No.	Course of the experiment
1	The experimental aim was to study the response of the culture when heavy overfeeding and consequently high specific growth rate occurs. The sequence of predetermined and real metabolic states was MS = {MS3(20h), MS5(4h)}. Overfeeding caused an increase in liquid-phase EtOH up to 40 g L^{-1}.
2	Similar control to that in Experiment 1. The sequence of metabolic states was MS = {MS5(0.5h), MS3(23.5h)}. Serious overfeeding took place with an increase of EtOH up to 25 g L^{-1}.
3	Control carried out as in Experiment 1. The sequence of predetermined and real metabolic states was MS = {MS3(20h), MS4(4h)}.
4	In this experiment the sequence of metabolic states was MS = {MS3(21h), MS4(3h)}.
5	A predetermined sequence of the metabolic states MS = {Border State 2,3,4} was examined. The sequence of actual metabolic states was MS = {MS5(1h), MS0÷MS1(8h), MS2(2h), MS3(4.5h), MS2(1h), MS0(2h), MS4(3.5h), MS5(2h)}. The states MS0 and MS2 were created by splitting the Border State 2, see the text of the article for details. There was complete utilisation of substrate.
6	A predetermined sequence of the metabolic states MS = {Border State 2,3,4,5} was examined. The actual sequence of metabolic states was MS = {MS1(2h), MS2÷MS3(6h), MS0(1h), MS2(2h), MS3(6h), MS4(4h), MS5(3h)}. There was complete utilisation of substrate.
7	A predetermined sequence of the metabolic states MS = {Border State 2,3,5} was examined. The actual sequence of metabolic states was MS = {MS5÷MS4(3h), MS2÷MS3(6h), MS3(10h), MS5(5h)}. There was complete utilisation of substrate.
8	A predetermined sequence of the metabolic states MS = {1,0,3,1,0,3} was examined. The actual sequence of metabolic states was MS = {MS1(3h), MS0(13.5h), MS2(1h), MS3(2h), MS4(1h), MS1(0.5h), MS0(1h), MS3(1h), MS4÷MS5(1h)}. A dramatic decline of the ergosterol purity appeared during the long-lasting oxidative part MS0 of the original border state.
9	The experiment was performed with the aim of repeating experiment 8 to verify the behaviour of the culture in the oxidative part of the original border state concerning purity of ergosterol. Therefore the actual sequence of metabolic states was MS = {MS5÷MS4(2.5h), MS0(21.5h)}. This experiment confirmed the decline of ergosterol purity during MS0 state.

10 The main aim of the experiment was to test the knowledge-based system. Therefore the predetermined sequence of metabolic states, MS = {1,0,3,4,0,3,4} was examined. The actual sequence of metabolic states was then MS = {MS5÷MS4(1.5h), MS1(2h), MS0(5h), MS3(2.5h), MS4(1.5h), MS0(5.5h), MS3(3h), MS4(3h)}.

11 The sequence of metabolic states MS = {3(11h), 4(5h), 3(6h), 4(2h)} was chosen as a new control strategy. The actual sequence of metabolic states was MS = {MS3÷MS4(3h), MS3(3h), MS1(2h), MS3(3.5h), MS4(5h), MS3(5.5h), MS4(2h)}.

Table 3

The final version of the metabolic states considered

MS	Characteristics
MS0	oxidative (glucose only) – border
MS1	oxidative (glucose only)
MS2	oxidative-fermentative – border
MS3	oxidative-fermentative
MS4	oxidative (glucose +ethanol)
MS5	oxidative (ethanol only)
MS6	starvation

Table 4

Results from experiments 1 – 11

Exp. No.	μ h^{-1}	Erg_0 %	Erg_m %	Erg_f %	$Y_{X/S}$ g g^{-1}	p g L^{-1} h^{-1}	$Y_{X/S} \cdot p$ g L^{-1} h^{-1}	$Y_{erg/S}$ g g^{-1}	p_{erg} g L^{-1} h^{-1}	$Y_{erg/S} \cdot p_{erg}$ g L^{-1} h^{-1}	X_f g L^{-1}	erg_f mg L^{-1}	erg_{pur} %
1.	0.17 – 0.01	0.3	1.2	0.93	0.110	0.47	0.052	0.0010	0.0049	5.01×10^{-6}	13.10	121.83	93
2.	0.20 – 0.02	0.1	1.3	0.97	0.113	0.37	0.042	0.0011	0.0041	4.49×10^{-6}	10.43	101.17	92
3.	0.11 – 0.04	0.8	1.6	1.61	0.161	0.23	0.037	0.0026	0.0052	13.48×10^{-6}	8.5	136.85	92
4.	0.13 – 0.02	1.1	2.0	1.40	0.221	0.25	0.055	0.0031	0.0037	11.45×10^{-6}	7.16	100.24	86
5.	0.09 – 0.02	1.0	2.3	2.07	0.372	0.18	0.067	0.0077	0.0047	36.19×10^{-6}	6.0	124.20	77
6.	0.15 – 0.01	1.2	2.2	1.45	0.358	0.32	0.115	0.0052	0.0049	25.44×10^{-6}	9.98	144.71	75
7.	0.09 – 0.03	0.8	2.8	2.23	0.325	0.17	0.055	0.0072	0.0048	34.79×10^{-6}	5.64	125.77	79
8.	0.10 – 0.03	1.0	1.8	1.79	0.367	0.36	0.132	0.0066	0.0080	52.55×10^{-6}	13.42	240.22	67
9.	0.06 – 0.02	1.1	1.6	0.92	0.241	0.31	0.075	0.0024	0.0025	5.54×10^{-6}	12.04	110.77	65
10.	0.10 – 0.02	1.0	2.3	1.63	0.394	0.53	0.209	0.0064	0.0099	63.58×10^{-6}	17.45	284.44	80
11.	0.10 – 0.03	1.0	2.4	2.43	0.283	0.55	0.156	0.0069	0.0151	103.84×10^{-6}	16.20	393.66	86

Table 5

Changes in the ergosterol purity and the content of ergosterol within individual metabolic states

	MS0	MS1	MS2	MS3	MS4	MS5	MS6
erg$_{pur}$							
DOWN	92 %	64 %	76 %	45 %	52 %	51 %	53 %
STEADY	8 %	36 %	24 %	29 %	45 %	14 %	22 %
UP	0 %	0 %	0 %	27 %	3 %	35 %	25 %
UP+STEADY	8 %	36 %	24 %	55 %	48 %	49 %	47 %
Erg							
DOWN	19 %	15 %	8 %	20 %	6 %	36 %	64 %
STEADY	34 %	7 %	27 %	21 %	40 %	13 %	36 %
UP	47 %	77 %	65 %	60 %	54 %	51 %	0 %
UP+STEADY	81 %	85 %	92 %	80 %	94 %	64 %	36 %
TOTAL	5965	1892	1897	15517	4140	2457	183