Research Signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

Recent Res. Devel. Biotech. & Bioeng., 5(2003): 65-78 ISBN: 81-271-0011-0

6 An integrated approach for cheese whey lactose valorisation

Lucília Domingues[1], Nelson Lima[2] and José A. Teixeira[3]
[1]Centro de Engenharia Biológica-IBQF, Universidade do Minho, Campus de Gualtar 4710-057 Braga, Portugal; [2]Centro de Engenharia Biológica-IBQF, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal; [3]Centro de Engenharia Biológica-IBQF, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal

Abstract

The development of any fermentation process requires the integration of life and engineering sciences with the aim of obtaining a high productivity system. The use of immobilized cells is, without any doubt, one of the most interesting ways to achieve this purpose, being several the advantages they present. Moreover, they are a clear example of how life and engineering sciences interact in the development of a fermentation process.

As the cost of the biomass support material is known to be a limiting factor in the implementation of a continuous high-cell-density fermentation process, the use of low cost immobilization technique is required as is the case of the application of yeast flocculating cells. Substrate must also be available in large amounts and at low cost, as is the case of lactose from cheese whey, a by-product of dairy industry.

Correspondence/Reprint request: Prof. José A. Teixeira, Centro de Engenharia Biológica-IBQF. Universidade do Minho Campus de Gualter, 4710-057 Braga, Portugal. E-mail: jateixeira@deb.uminho.pt

Results are presented on the development of continuously operating high productivity systems using flocculating yeast cells for the production of ethanol and/or β-galactosidase using lactose from cheese whey as fermentation substrate. The presentation of the results is divided into three main parts – the first one deals with a brief introduction on the state of the art of the different subjects related hereby, namely, the cheese whey problematic, the construction of lactose metabolising S. cerevisiae cells and the use of flocculation in continuous high-cell-density systems. The second part presents results on the construction, using molecular biology techniques, of yeast strains with the ability to form flocs and perform the desired biotransformation being it the production of ethanol or β-galactosidase. The third one deals with the operation, at laboratory scale, of continuous high cell density airlift bioreactors using the previously developed yeast strains.

1. Introduction

1.1. The cheese whey problematic

Cheese whey is a major pollutant and solutions for its disposal are needed. Drying is one of the solutions that have been considered and industrially employed but more profitable solutions are required. Whey protein concentrates (WPC) production has been increasing and WPC is nowadays the third major product obtained from cheese whey [1]. When obtaining WPC, typically by ultrafiltration, a lactose rich fraction – the permeate - is obtained that remains a major pollutant and a profitable use must be found for it. The dairy industry is left with the paradox between a need to recover lactose as a food ingredient and an increasing consumer demand for lactose-free dairy products [2]. The future trend for cheese factories will be to move towards zero discharge, that is, move away from high disposal costs and find more environmentally friendly and profitable applications for lactose [2]. Lactose fermentation is certainly one of the alternatives. Lactose-containing whey streams could be used as abundant and renewable raw material for whey

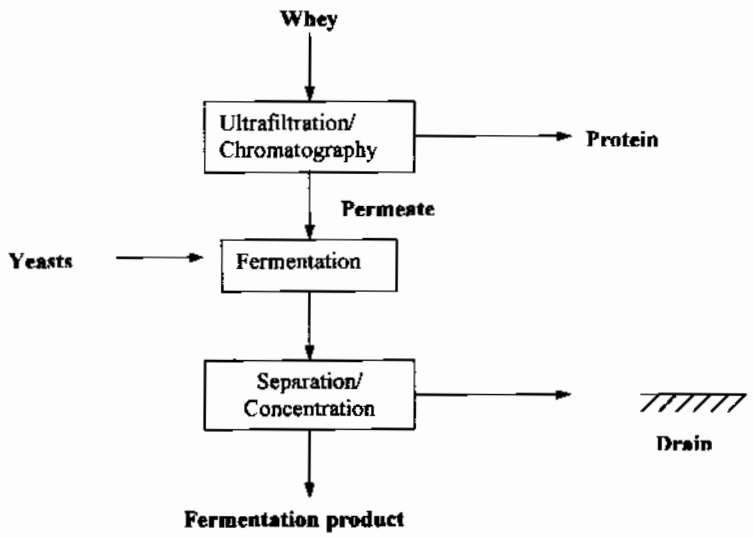

Figure 1. Main process units for cheese whey valorization.

fermentation, where the role of lactose would be to provide the carbohydrate source. A simplified flow sheet of such a process is presented in Figure 1. With this procedure, the objectives of valorisation of a by-product of cheese production and the reduction of the organic load associated with its disposal can be achieved. Two added value products – one obtained from lactose fermentation and a protein concentrate with good nutritional and functional properties – can be obtained. Also, a 90% reduction in the polluting organic load of cheese whey can be obtained. To make this alternative use for lactose attractive, the development of fermentation processes with increased productivity must be considered. Existing processes can be improved and new processes developed if a high productivity continuous process is implemented. High cell density continuous systems, keeping high cellular concentrations inside the bioreactor, are known for its high productivity. Among these, the ones that use flocculent cells are surely attractive due to its simplicity and low cost.

1.2. Construction of lactose metabolising *S. cerevisiae* cells

Some microorganisms are natural lactose consumers, being the genes responsible for lactose metabolisation potential candidates for cloning in *S. cerevisiae* cells. The bacteria *Escherichia coli* was one of the first microorganisms to be studied in respect to lactose genetics. Three different approaches can be utilized to express the *E. coli lacZ* gene in *S. cerevisiae*. As β-galactosidase is citosolic, lactose has to be transported to the cytoplasm in order to occur its hydrolysis. As a consequence, the *lacY* gene coding for the lactose permease should also be cloned together with the β-galactosidase gene in order to obtain *S. cerevisiae* cells able of utilizing lactose. However, when cloning the *lac* operon in *S. cerevisiae* in a multicopy plasmid it was not possible to obtain transformants able of utilizing lactose [3]. The yeast transformants even expressing β-galactosidase were not able to grow on lactose due to the non-funcionality of the *E. coli* lactose transport system. The second approach used was to secrete the *E. coli* β-galactosidase in *S. cerevisiae* cells. For that, several signal sequences have been tried, namely from the genes *SUC2* [4], *Mfα* [5] and *STA2* [6], but these attempts were unsuccessful. More recently, the fusion of glucoamylase residues with *E. coli* β-galactosidase was shown to facilitate its secretion [7]. However, the secretion was not as efficient as with the glucoamylase enzyme. Also, using the signal sequence of the membranar protein Ggpl, it was possible to direct the *E. coli* β-galactosidase to the extracellular membrane [8]. The third approach described in the literature deals with the spontaneous lysis of yeast cells overproducing the *E. coli* β-galactosidase enzyme [9].

The utilization of lactose by *Kluyveromyces* strains consists in a lactose transport system together with an intracellular β-galactosidase [10]. Being so, two different strategies can be designed: to clone both the lactose permease and β-galactosidase genes or to direct the β-galactosidase production to the extracellular medium. As the lactose transport system from *Kluyveromyces* is eucariotic, it is more prone to work in *S. cerevisiae* than the *E. coli* one. With the first approach it was possible to obtain *S. cerevisiae* cells growing on lactose [11, 12, 13]. The second approach, that is, to secrete *Kluyveromyces* β-galactosidase was not successful achieved [14]. More recently, the construction of recombinant *S. cerevisiae* strains secreting *Kluyveromyces* β-galactosidase has been reported [15, 16]. For that, the *LAC4* gene was fused in frame to the yeast α-

factor secretion signal and expressed under the control of the yeast *ADH2* promoter and *CYC1* terminator [15, 16].

The filamentous fungi *Aspergillus niger* is a great producer of several secreted glycoproteins, some used in industrial processes. Among these is β-galactosidase, mainly used to hydrolyse lactose in acid whey [17]. The cloning of the *lacA* gene (coding for *A. niger* β-galactosidase) with its own signal sequence resulted in recombinant *S. cerevisiae* cells secreting β-galactosidase [18, 19, 20, 21].

1.3. Flocculation and continuous high-cell-density systems

Yeast flocculation, defined as the non-sexual cell aggregation, allows for cell separation from the fermented broth. It is one of the most important properties of yeast strains used in traditional processes like brewing and winemaking. Yeast flocculation can be of great interest in modern biotechnology, namely when using high-cell-density systems [22], as operation at high cell density allows for overall increased productivity. By using flocculent cells, the first step in downstream processing is greatly simplified with cells easily separated from the culture medium, just by stopping agitation and/or aeration.

The use of flocculent cells also allows for the continuous operations of bioreactors at high cell density that have as main characteristics [22]:

- high cell densities per unit bioreactor volume, resulting in very high fermentation rates;
- reuse of the same biocatalyst (cells) for extended periods of time due to constant cell regeneration;
- possibility of operation beyond the washout rate;
- easy separation of biocatalyst (cells) from the liquid phase;
- minimised risk of contamination;
- smaller bioreactor volumes, reducing capital costs.

Continuous fermentation systems using flocculent yeast cells can be economically more attractive as there is no need for the immobilisation support. This is a clear advantage over other immobilisation techniques since it is well known that support represents a major cost in immobilisation procedures.

2. Metabolic engineering for direct lactose utilization by flocculent *S. cerevisiae*

The development of flocculent yeast cells for ethanol or protein production from cheap raw materials, namely, cheese whey, as a way of increasing overall productivity of these systems [23] is discussed below.

2.1. Construction of new flocculent yeast strains

When considering the genetic transformation of flocculent yeast cells, some additional difficulties may be expected, due to its different cell wall structure. It is well known that flocculent yeast cells are more resistant to protoplast formation than non-flocculent yeast cells [24]. In what concerns the brewing yeasts, several new properties have been introduced with recombinant DNA methods [25]. However, it is worth noting that this type of yeasts will only flocculate at the end of fermentation. Thus, as

fermentation, where the role of lactose would be to provide the carbohydrate source. A simplified flow sheet of such a process is presented in Figure 1. With this procedure, the objectives of valorisation of a by-product of cheese production and the reduction of the organic load associated with its disposal can be achieved. Two added value products – one obtained from lactose fermentation and a protein concentrate with good nutritional and functional properties – can be obtained. Also, a 90% reduction in the polluting organic load of cheese whey can be obtained. To make this alternative use for lactose attractive, the development of fermentation processes with increased productivity must be considered. Existing processes can be improved and new processes developed if a high productivity continuous process is implemented. High cell density continuous systems, keeping high cellular concentrations inside the bioreactor, are known for its high productivity. Among these, the ones that use flocculent cells are surely attractive due to its simplicity and low cost.

1.2. Construction of lactose metabolising *S. cerevisiae* cells

Some microorganisms are natural lactose consumers, being the genes responsible for lactose metabolisation potential candidates for cloning in *S. cerevisiae* cells. The bacteria *Escherichia coli* was one of the first microorganisms to be studied in respect to lactose genetics. Three different approaches can be utilized to express the *E. coli lacZ* gene in *S. cerevisiae*. As β-galactosidase is citosolic, lactose has to be transported to the cytoplasm in order to occur its hydrolysis. As a consequence, the *lacY* gene coding for the lactose permease should also be cloned together with the β-galactosidase gene in order to obtain *S. cerevisiae* cells able of utilizing lactose. However, when cloning the *lac* operon in *S. cerevisiae* in a multicopy plasmid it was not possible to obtain transformants able of utilizing lactose [3]. The yeast transformants even expressing β-galactosidase were not able to grow on lactose due to the non-funcionality of the *E. coli* lactose transport system. The second approach used was to secrete the *E. coli* β-galactosidase in *S. cerevisiae* cells. For that, several signal sequences have been tried, namely from the genes *SUC2* [4], *Mfα* [5] and *STA2* [6], but these attempts were unsuccessful. More recently, the fusion of glucoamylase residues with *E. coli* β-galactosidase was shown to facilitate its secretion [7]. However, the secretion was not as efficient as with the glucoamylase enzyme. Also, using the signal sequence of the membranar protein Ggpl, it was possible to direct the *E. coli* β-galactosidase to the extracellular membrane [8]. The third approach described in the literature deals with the spontaneous lysis of yeast cells overproducing the *E. coli* β-galactosidase enzyme [9].

The utilization of lactose by *Kluyveromyces* strains consists in a lactose transport system together with an intracellular β-galactosidase [10]. Being so, two different strategies can be designed: to clone both the lactose permease and β-galactosidase genes or to direct the β-galactosidase production to the extracellular medium. As the lactose transport system from *Kluyveromyces* is eucariotic, it is more prone to work in *S. cerevisiae* than the *E. coli* one. With the first approach it was possible to obtain *S. cerevisiae* cells growing on lactose [11, 12, 13]. The second approach, that is, to secrete *Kluyveromyces* β-galactosidase was not successful achieved [14]. More recently, the construction of recombinant *S. cerevisiae* strains secreting *Kluyveromyces* β-galactosidase has been reported [15, 16]. For that, the *LAC4* gene was fused in frame to the yeast α-

factor secretion signal and expressed under the control of the yeast *ADH2* promoter and *CYC1* terminator [15, 16].

The filamentous fungi *Aspergillus niger* is a great producer of several secreted glycoproteins, some used in industrial processes. Among these is β-galactosidase, mainly used to hydrolyse lactose in acid whey [17]. The cloning of the *lacA* gene (coding for *A. niger* β-galactosidase) with its own signal sequence resulted in recombinant *S. cerevisiae* cells secreting β-galactosidase [18, 19, 20, 21].

1.3. Flocculation and continuous high-cell-density systems

Yeast flocculation, defined as the non-sexual cell aggregation, allows for cell separation from the fermented broth. It is one of the most important properties of yeast strains used in traditional processes like brewing and winemaking. Yeast flocculation can be of great interest in modern biotechnology, namely when using high-cell-density systems [22], as operation at high cell density allows for overall increased productivity. By using flocculent cells, the first step in downstream processing is greatly simplified with cells easily separated from the culture medium, just by stopping agitation and/or aeration.

The use of flocculent cells also allows for the continuous operations of bioreactors at high cell density that have as main characteristics [22]:

- high cell densities per unit bioreactor volume, resulting in very high fermentation rates;
- reuse of the same biocatalyst (cells) for extended periods of time due to constant cell regeneration;
- possibility of operation beyond the washout rate;
- easy separation of biocatalyst (cells) from the liquid phase;
- minimised risk of contamination;
- smaller bioreactor volumes, reducing capital costs.

Continuous fermentation systems using flocculent yeast cells can be economically more attractive as there is no need for the immobilisation support. This is a clear advantage over other immobilisation techniques since it is well known that support represents a major cost in immobilisation procedures.

2. Metabolic engineering for direct lactose utilization by flocculent *S. cerevisiae*

The development of flocculent yeast cells for ethanol or protein production from cheap raw materials, namely, cheese whey, as a way of increasing overall productivity of these systems [23] is discussed below.

2.1. Construction of new flocculent yeast strains

When considering the genetic transformation of flocculent yeast cells, some additional difficulties may be expected, due to its different cell wall structure. It is well known that flocculent yeast cells are more resistant to protoplast formation than non-flocculent yeast cells [24]. In what concerns the brewing yeasts, several new properties have been introduced with recombinant DNA methods [25]. However, it is worth noting that this type of yeasts will only flocculate at the end of fermentation. Thus, as

transformation is done in exponential growing cells, they can be transformed with DNA at a non-flocculent state. This will not be the case for constitutively flocculent yeast cells.

2.2. Construction of auxothrophic mutants and transformation of flocculent yeast cells

One of the pre-requisites for transformation of an organism with DNA is a selective marker that will allow for identification of the recombinant clones.

UV radiation was used to construct *ura*$^-$ mutants from a respiratory competent and highly flocculent *S. cerevisiae* NCYC869 strain [26]. The auxothrophic mutants were selected by growth on minimal medium with glycerol as carbon source supplemented with uracil and 1mg/mL 5-fluoro-orotic acid (5-FOA). The mutant A3 was selected as it showed the best back mutation frequency ($<6.5x10^{-10}$) and produced the same degree of flocculation. The A3 mutant was affected in the genes either coding for orotidine-5'-phosphate decarboxylase (*ura3*) or for orotidine -5'-phosphate pyrophosphorylase (*ura5*). The mutation was confirmed to be *ura3* by genetic complementation with a plasmid containing the URA3 marker [27]. For the flocculent yeast transformation, the lithium acetate method was preferred. It should be noted that in contrast to spheroplast transformation, method that implies cell wall regeneration, the lithium acetate method avoids perturbation of cell walls. Therefore the flocculation capacity, which is a cell wall dependent phenomenon, is less prone to be affected by the lithium acetate method. In fact, the flocculence ability of yeasts was not affected by the uptake of foreign DNA [27]. Moreover, lithium is one of the ions known to have an inhibitory effect on yeast flocculation. Lithium ions are less potent inhibitors than sodium ions [28], but depending on the concentration and yeast strain they can affect flocculation. This is extremely advantageous in what concerns transformation of constitutively flocculent yeast strains. While making yeast cells competent for receiving DNA, lithium also destabilizes the yeast flocs, facilitating the transformation of flocculent yeast cells.

2.3. Introducing new properties in the flocculent *S. cerevisiae* NCYC869-A3 strain-ethanol production from lactose

Aiming at producing ethanol from lactose-composed raw materials with increased productivity, a flocculent *S. cerevisiae* strain fermenting lactose was constructed [13]. The auxotrophic mutant *S. cerevisiae* NCYC869-A3 was used in a co-transformation procedure using plasmid KR1B-Lac4-1 [11] together with a linear fragment from pYAC4 [13]. The plasmid KR1B-Lac4-1 harboured the *LAC4* (coding for β-galactosidase) and *LAC12* (coding for lactose permease) genes of *Kluyveromyces marxianus*. The expression of these two genes allowed for lactose metabolisation in the recombinant *S. cerevisiae* strain (Figure 2). The linear YAC4 fragment harboured the *URA3* gene, used as a selective marker for recombinants. The selective medium used was 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal)/YNB/galactose. In this medium, only the clones that have the *URA3* gene incorporated are able to grow. On the other hand, not all *ura*$^-$ clones will be *Lac*$^+$ transformants, so Xgal blue/white colour screen was used to identify clones with *LAC4* gene positive expression. This approach enabled the selection of *Ura*$^+$ clones and the identification for positive *Lac*$^+$ transformants. From a total of 1212 transformants only 4 clones had the blue colour indicative of β-galactosidase. From these 4 clones, only 2, named T1 and T2, kept a stable *Lac*$^+$ phenotype. The transformants were thereafter kept in

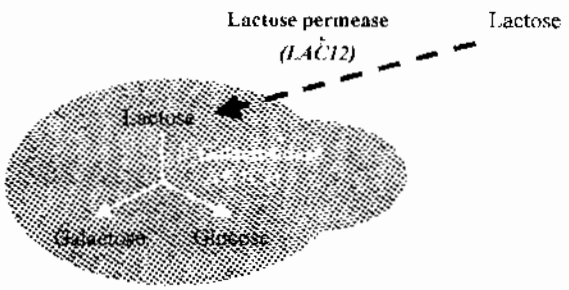

Figure 2. Illustration of the construction of the flocculent strain *S. cerevisiae* NCYC869-A3/T1.

YNB/lactose medium, that is, imposing a double selection pressure (Figure 3).

Both transformants, T1 and T2, gave a positive colour reaction on screening for β-galactosidase activity [29]. Some modifications of the cell morphology of transformant T2 were observed. Both transformants presented some cells bigger than the traditional *S. cerevisiae* cell; the T2 transformant also presented bizarre forms and pseudo-mycelium and for that reason was not considered for further studies. The location of pKR1B-Lac4-1 as a plasmid in the recombinant strain was confirmed after restriction digestion analysis of the original pKR1B-Lac4-1 and plasmids isolated from the recombinant strain T1. Also the low stability of these transformants in non-selective medium indicated the presence of the pKR1B-Lac4-1 in its autonomous replicating form.

Figure 3. Phenotypic response of T1 and T2 transformants to the selective medium YNB-Xgal-lactose; controls for *S. cerevisiae* (Sc) and *K. marxianus* (Km) [30].

The recombinant strain T1 was able to grow and ferment the substrate lactose. Even though the recombinant strain was able to flocculate, it presented a different behaviour from the host strain *S. cerevisiae* NCYC869-A3. The flocculation ability from the recombinant strain was more sensitive to environmental conditions than that of the host strain, loosing its ability to flocculate more easily. This observed flocculation instability could be due to the lactose permease cloning, a cell membrane protein. Because flocculation is a cell wall phenomenon, production of a new protein in the cell membrane may interfere with cell-to-cell interaction. Supporting this, the transformation of the same host strain, the mutant A3, with a plasmid harbouring an extracellular *A. niger* β-

galactosidase, resulted in a recombinant flocculent *S. cerevisiae* strain with the same flocculation properties as the host strain [20, 21].

The recombinant strain hereby described is able to metabolise and ferment lactose in a way comparable to that of the existing lactose-fermenting yeast strains. The construction of a good flocculent lactose-fermenting *S. cerevisiae* strain raises new perspectives for ethanol production from lactose-composed raw materials. Even though the flocculation capacity of the recombinant strain showed some instability, this could be overcome by a selective bioreactor operation for the flocculent cells [31, 32], as described below.

2.4. Extracellular protein production by flocculent yeast cells

Besides ethanol production, high-cell-density systems could also be attractive for protein production, especially for extracellular proteins. In this way, both system productivity and protein separation are highly enhanced. Taking this into account, a new system for yeast strain construction was designed, were β-galactosidase is now secreted into the culture medium and lactose is hydrolysed in the cell surroundings (Figure 4). However, the use of flocculent yeast cells for extracellular protein production could be hindered by diffusion limitations in cell flocs. With the aim of studying heterologous protein secretion by flocculent yeast cells, both flocculent and non-flocculent *S. cerevisiae* strains secreting *A. niger* β-galactosidase were constructed [20]. The yeast strains used as host strains for transformation had the same genetic background except for the integration of *FLO1* gene in the flocculent strain. Being so, the recombinant strains constructed allow for comparison of protein production by flocculent and non-flocculent strains having otherwise the same genetic background.

In order to express the *A. niger* β-galactosidase in yeast, the β-galactosidase encoding gene *lacA* was coupled to the *ADH1* promotor on a multicopy plasmid carrying also the selectable marker gene for copper resistance, *CUP1*. The constructed plasmid was named pLD1 [18]. Two brewer's yeast strains, W204, and the highly flocculent derivative strain, W204-FLO1L(INT) [33], were transformed with pLD1 and copper resistant transformants were selected.

As referred above, in this case no differences in the flocculation ability between host and recombinant strains were observed. Confirming that the flocculation instability

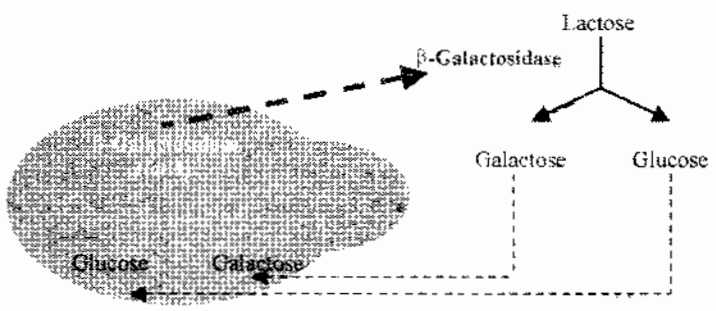

Figure 4. Illustration of the construction of the two brewer's yeast strain (the non-flocculent strain W204 and the flocculent derivative W204-FLO1L(INT)) and flocculent strain *S. cerevisiae* NCYC869-A3/pVK1.1.

observed with the recombinant strain *S. cerevisiae* NCYC869-A3/T1 should be due to the lactose permease gene cloning.

β-Galactosidase activity was detected in the culture supernatant of the yeast transformants but not in the supernatant of the host strains. The results clearly showed that the recombinant brewer's yeast secretes active β-galactosidase into the culture medium. When comparing the two transformant strains, the non-flocculent W204/pLD1 and the flocculent W204/FLO1L(INT)/pLD1, no significant differences between the β-galactosidase activity were detected in the supernatants. A slight β-galactosidase accumulation inside the floc was observed but it was suggested that at the phase of highest production, β-galactosidase accumulation occurred inside the floc and that the protein was able to diffuse through the porous floc afterwards [20]. Using polymers that increase floc porosity [34] the β-galactosidase accumulation could be minimised.

Using the dominant marker *CUP1* allowed for the transformation of industrial strains. The recombinant strains secreted β–galactosidase into the culture medium and grew on lactose as the sole carbon source. However, when grown on lactose, recombinant strains presented rather slow duplication times (5 hours). Moreover, at a lactose concentration higher than 10g/L, glucose and galactose accumulated in the culture medium.

Nevertheless, the results obtained with the construction of these recombinant strains [20], are encouraging, in terms of the secretion of biological macromolecules by flocculent cells since, for the large β-galactosidase, no significant differences were found between flocculent and non-flocculent cells.

2.5. Introducing new properties in the flocculent *S. cerevisiae* NCYC869-A3 strain-β-galactosidase production from lactose

As the recombinant brewer's yeast strains described above lack biotechnological application due to their low growth phenotype in lactose medium, but have proved the feasibility of using flocculent yeast cells for extracellular protein production, improved recombinant flocculent *S. cerevisiae* strains secreting β-galactosidase were constructed.

The flocculent *ura3*⁻ *S. cerevisiae* NCYC869-A3 strain, previously used as host strain for construction of the recombinant strain T1, was transformed with the vector pVK1.1 [18] containing the *A. niger* β-galactosidase-encoding gene under the *ADH1* promoter [21]. The transformants were selected in YNB/galactose medium containing Xgal. All the recombinant colonies obtained were blue. The flocculation ability of the recombinant strain was identical to that of the host strain [21]. When compared to the previously constructed strain, a 20-fold increase of β-galactosidase activity was detected in the supernatant, for the same culture conditions. As a consequence, the metabolism of 10g/L lactose occurred in less than 24 hours, while for the previously constructed recombinant brewer's strains 150 hours were needed. Moreover, the recombinant strain *S. cerevisiae* NCYC869-A3/pVK1.1 metabolised 50g/L lactose in less than 24 hours.

For a better characterisation of the recombinant strain, fermentations were done under controlled conditions in a 2-L bioreactor [21]. The semi-synthetic medium SSlactose with 2 g/L yeast extract and cheese whey permeate were used as substrate. In

all the fermentations, stability studies of the plasmid indicated that more than 75% of the population retained the plasmid at the end of the culture period. At this time colonies containing the plasmid were identified by their blue phenotype on 2% lactose/galactose minimal agar plates containing Xgal [21]. In bioreactor culture, when using an initial lactose concentration of 100g/L in the fermentation medium [21], there was 300-fold increase in β-galactosidase activity compared with the amount detected using the previously constructed strains in shake-flask culture [20]. When using cheese-whey permeate (initial lactose concentration 50g/L) from a Portuguese dairy industry as a substrate, in less than 40 hours all the lactose was metabolised, producing 2,635U extracellular β-galactosidase activity mL^{-1} [21]. Ethanol production was also observed.

In what concerns the β-galactosidase produced, it retains the main characteristics of *A. niger* β-galactosidase, namely pH and temperature optima. The recombinant enzyme is glycosylated and is secreted in a rather pure form as observed from protein electrophoresis analysis. These aspects are very important from the biotechnological point of view. The maintenance of the *A. niger* β-galactosidase pH optimum makes this enzyme particularly suitable for acid whey hydrolysis. Moreover, the hydrolysis can be made at relatively high temperatures minimizing the risk of microbial contamination. The fact that the enzyme is produced in a rather pure form together with the cells flocculence, greatly facilitates the downstream processing of the produced enzyme. To confirm the viability of using the recombinant enzyme in cheese whey hydrolysis, the produced enzyme was applied to the cheese whey permeate hydrolysis, as shown in Figure 5 [23]. In just 11 hours 74% of the lactose present in cheese whey was hydrolysed while at 21 hours this value increased to 90% [21, 23].

The flocculent strain *S. cerevisiae* NCYC869-A3/pVK1.1 constructed for *A. niger* β-galactosidase secretion is clearly superior to other, previously constructed strains. Moreover, besides being a good producer of extracellular β-galactosidase, produces ethanol and metabolises lactose, important aspects for biotechnological application.

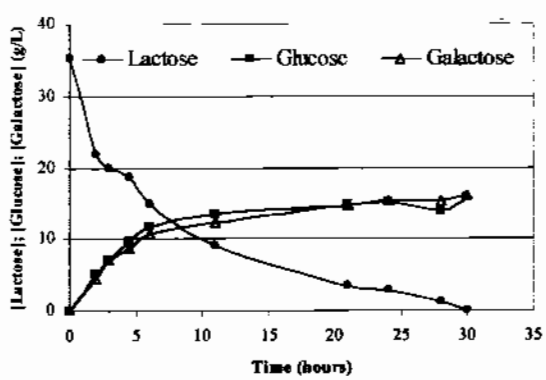

Figure 5. Application of the produced β-galactosidase to cheese whey permeate hydrolysis. The fermentation supernatant was treated with an ultrafiltration membrane (Centricon-50 membranes, cut-off 50KDa) and applied directly to the cheese-whey without further treatment (0.5mL β-galactosidase sample to 10mL cheese-whey permeate).

3. Application of the constructed strains to continuous high-cell-density systems

The main purpose on constructing new flocculent yeast strains, namely with the property of metabolising lactose, was for one side to apply to the bioremediation of cheese whey permeate and on the other side to apply to continuous high-cell-density systems aiming at obtaining higher overall productivity (in ethanol or/and in β-galactosidase). In cheese whey, the main component responsible for the high BOD (Biochemical Oxygen Demand) and COD (Chemical Oxygen Demand) is lactose, as the protein recovery reduces the COD of the whey only about 10g/L [35]. Therefore, the development of a high-productivity lactose fermenting process is of prime importance.

3.1. Ethanol production from lactose

Alcohol fermentation of lactose was investigated using the constructed flocculent strain *S. cerevisiae* NCYC869-A3/T1. After the adaptation period referred above, it was observed that this recombinant strain metabolised 50g/L lactose in less than 40 h, producing 16g/L ethanol representing an ethanol productivity of $0.45 gL^{-1}h^{-1}$ [36]. These preliminary results indicated that the constructed strain could be applied to continuous high-cell-density systems if the flocculation instability could be overcome [13]. Continuous operation in a bioreactor with an adequate design [32] can be used to select for most flocculating cells from a mixed culture, allowing for the possibility of accumulating a high biomass concentration in the bioreactor with the inherent advantages of operating as a continuous high-cell-density system. The need for selection for the most flocculating cells led to the choice of using and airlift bioreactor, which exhibits low shear stresses due to the absence of mechanical agitation [36]. The existence of the sedimentation zone in the top of the reactor coupled with the semicylindrical fence in the outlet region [36], helped in the retention of flocculent cells, the non-flocculent cells being washed-out. After just 13 days of continuous operation, selection of a 100% flocculent culture was possible [36]. With this system, an ethanol productivity of $11 gL^{-1}h^{-1}$ was obtained (with a feed lactose concentration of 50g/L and a dilution rate of $0.55 h^{-1}$), being seven-fold larger than the one in conventional continuous systems. Moreover, the ethanol productivity obtained with this system is clearly higher than the one obtained with other processes based on metabolically engineered *S. cerevisiae* cells (Table 1). The operation of the continuous bioreactor was maintained for 6 months, in order to test its stability. During all the period of operation the cell viability varied from 70% to 99% [36]. Plasmid stability was also monitored throughout the continuous operation and found to be very similar to cell viability [36]. As

Table 1. Ethanol productivity from lactose using genetically modified *S. cerevisiae* cells.

Ethanol productivity ($gL^{-1}h^{-1}$)	Reference
0.1-0.2	[9]
0.15	[16]
0.139-0.4	[19]
1.0	[37]
11	[36]

the lactose is the only carbon source present in the culture medium, cells have to maintain the plasmid otherwise they will die. In other words, lactose functions as positive selective pressure to maintain the plasmid. In 6 months operation this system proved to be stable with total conversion of lactose and constant ethanol conversion yield [36].

One of the main advantages attributed to the continuous process, in general, is the risk of contamination with faster growing microorganisms and consequent takeover of the culture. However, when working at high-cell-density continuous systems it is generally observed a good resistance of these systems to nonflocculent contaminants (due to continuous selection for flocculent cells). The high-cell-density continuous airlift bioreactor operating with the recombinant flocculent strain *S. cerevisiae* NCYC869-A3/T1 was deliberately contaminated with a faster growing microorganism and proved to be resistant to contamination [38]. The results obtained in this experiment support the argument of increased resistance to nonflocculent contaminants in the bioreactor operating at sufficiently high flow rates [38].

The contamination assay was set-up with the continuous bioreactor operating at $0.45h^{-1}$ dilution rate. The contaminant was a recombinant *E. coli* strain transformed with the pGLO plasmid which harboured the gene coding for GFP (Green Fluorescent Protein). This recombinant *E. coli* strain was easily detectable and quantified as the fluorescence from GFP could be monitored. The inoculum of the artificial contaminant was introduced in the bioreactor operating at $0.45h^{-1}$ so that the initial bacterial concentration in the bioreactor was $1x10^7$cells/mL [38]. After 4 hours of operation, an accentuated decrease in contaminant concentration was observed in the bioreactor and in the effluent. This extremely low value of contaminant concentration remained constant for the next 20 hours, clearly indicating that, at high dilution rates, bacterial contamination is not a problem for these bioreactors [38].

3.2. Ethanol production from cheese whey permeate

Batch experiments showed that the recombinant *S. cerevisiae* NCYC869-A3/T1 strain was capable of utilizing cheese whey permeate as a carbon and energy source, fermenting 50g/L or 100g/L of lactose present in cheese whey permeate without any additional nutrient supply [39]. Moreover, the complete lactose metabolisation was observed in less than 40 hours, producing ethanol at 21g/L and 53g/L for initial lactose concentration of 50g/L and 100g/L, respectively. When operating in continuous high-cell-density system using cheese whey permeate as substrate, different experiment conditions were tested (dilution rate, feed lactose concentration). For cheese whey permeates with a lactose concentration of 50g/L, total lactose consumption was observed with a conversion yield of ethanol close to the expected theoretical value. Using a continuously operating 5.5-L bioreactor, ethanol productivity near $10gL^{-1}h^{-1}$ (corresponding to $0.45h^{-1}$ dilution rate) was obtained, which raises new perspectives for the economic feasibility of whey alcoholic fermentation [39]. Ethanol productivity is largely superior to that reached with conventional methods [40]. While producing ethanol, the recombinant *S. cerevisiae* strain cleared the cheese whey permeate of most organic substances, allowing for a significant reduction in the pollutant load of the cheese whey. When fed with concentrated cheese whey permeate (100g/L lactose) flocculation was strongly impacted, and it became impossible to reach the dilution rates obtained with semi-synthetic medium with 100g/L as deflocculated biomass was washed-out from the bioreactor [39]. The deflocculating effect

of concentrated cheese whey permeate was attributed to the salts concentration. If salts are removed, at least the same productivity as for the system fed with whey permeate can be expected, as confirmed by the data obtained with the continuous bioreactor fed with 100g/L semisynthetic medium [39]. Moreover, a 5% ethanol product is obtained, as shown for batch fermentation, minimizing the distillation costs [39].

Although batch ethanol production from whey is currently done in some countries [41], efforts have been made to improve the productivity of cheese whey permeate alcoholic fermentation. In Table 2, the highest ethanol productivities reported on literature are presented. It is clear that the developed continuous high-cell-density system is very interesting as an ethanol productivity of near $10gL^{-1}h^{-1}$ was obtained. This is more evident as there is still space for further development, once deflocculation has occurred when operating with concentrated cheese whey. If the concentrated cheese whey permeate is demineralised higher ethanol productivity can be expected.

Table 2. Systems for high ethanol productivity from cheese whey.

Organism	**Bioreactor operation type**	**Substrate**	**Ethanol productivity ($gL^{-1}h^{-1}$)**	**Ref.**
Kluyveromyces fragilis	Optimised batch process	Demineralised concentrated cheese whey permeate (CCWP) - 240g/L lactose	3.2	[42]
K. fragilis	Continuous process with cell recycling, $D=0.15h^{-1}$	CCWP - 100g/L lactose	7.1	[43]
K. fragilis	Immobilized continuous processes	Cheese whey	1.1	[44]
S. cerevisiae	*S. cerevisiae* cells coimmobilized with β-galactosidase	Cheese whey	4.5	[44]
S. cerevisiae	Continuous operation with cell recycling, $D=0.2h^{-1}$	CCWP - 150g/L lactose (*the CCWP has to be hydrolysed to glucose and galactose prior to use*)	13.6	[45]
Recombinant *S. cerevisiae*	Continuous high-cell-density operation with flocculent cells	Whey permeate – 50g/L lactose	10	[39]

3.3. β-galactosidase production from lactose/cheese-whey permeate

The bioreactor used for the continuous ethanol production from lactose with the recombinant strain *S. cerevisiae* NCYC869-A3/T1 was applied to the β-galactosidase production from lactose with the recombinant strain *S. cerevisiae* NCYC869-A3/pVK1.1. Experiments were done with lactose feed concentration of 50g/L and 100g/L. Overall, an increase in extracellular β-galactosidase productivity could be achieved when comparing

to batch systems (4 to 11 fold increase) [23]. The best result for β-galactosidase productivity –$6.2x10^5 UL^{-1}h^{-1}$- was obtained for the system operating at $0.24h^{-1}$ dilution rate and for a 50g/L feed lactose concentration. Together with extracellular β-galactosidase production an ethanol productivity of $9gL^{-1}h^{-1}$ was obtained for the bioreactor fed with $50gL^{-1}$ initial lactose concentration at $0.45h^{-1}$ dilution rate.

The developed system is particularly attractive for application in the dairy industry. Besides producing extracellular β-galactosidase the recombinant *S. cerevisiae* strain when applied to the airlift continuous bioreactor presents high ethanol productivity. The β-galactosidase can be easily separated by ultrafiltration and applied in the same industry (for preparation of lactose free products). Moreover, the pollutant load of cheese whey is greatly reduced.

These results demonstrate the feasibility of using continuous high-cell-density systems with flocculent yeast cells for extracellular production of heterologous proteins[23]. Up to now these systems have been mainly studied for the production of ethanol [22].

4. Conclusion

Yeast flocculation can be used to increase biotechnological systems productivity, namely for ethanol and extracellular protein production, using continuously operating high cell density bioreactors. The coupling of strain construction together with bioreactor development and the use of low cost substrates lead to improved biotechnological processes. However, further developments are needed before these systems can be applied at pilot/industrial scale. The processes that have been developed at laboratory scale have proved, without any doubt, that continuously operating airlift bioreactors with yeast flocculating cells are a very promising alternative for the production of low and medium added value fermentation products.

References

1. Horton, B. 1996, Whey Processing, May, 39.
2. Lifran, E.V., Hourigan, J.A., Sleigh, R.W., and Johnson, R.L. 2000, Food Australia, 52, 120.
3. Casadaban, M.J., Arias, A.M., Shapira, S.K., and Chou, J. 1983, Meth. Enzymology, 100, 293.
4. Das, R.C., Shultz, J.L., and Lehman, D.J. 1989, Mol. Gen. Genet., 218, 240.
5. Emr, S.D., Schauer, I., Hansen, W., Esmon, P., and Schekman, R. 1984, Mol. Cell Biol., 4, 2347.
6. Vanoni, M., Porro, D., Martegani, E., and Alberghina, L. 1989, Biochem. Biophys. Res. Commun., 164, 1331.
7. Venturini, M., Morrione, A., Pisarra, P., Martegani, W., and Vanoni, M. 1997, Mol. Microbiol., 23, 997.
8. Pignatelli, R., Vai, M., Alberghina, L., and Popolo, L. 1998, Biotechnol. Appl. Biochem., 27, 81.
9. Porro, D., Martegani, E., Ranzi, B.M., and Alberghina, L. 1992, Biotechnol. Bioeng., 39, 799.
10. Wray, L.V.Jr., Witte, M.M., Dickson, R.C., and Riley, M.I. 1987, Mol. Cell Biol., 7, 1111.
11. Sreekrishna, K. and Dickson R.C. 1985, Proc. Natl. Acad. Sci. USA 82, 7909.
12. Rubio-Teixeira, M., Castrillo, J.I., Adam, A.C., Ugalde, U.O., and Polaina, J. 1998, Yeast, 14, 827.
13. Domingues, L., Teixeira, J.A. and Lima, N. 1999, Appl. Microbiol. Biotechnol., 51, 621.
14. Becerra, M., Cerdán, E., and Siso, M.I.G. 1997, Biochim. Biophys. Acta, 1335, 235.
15. Becerra, M., Prado, S.D., Cerdán, E., and Siso, M.I.G. 2001, Biotechnol. Lett., 23, 33.
16. Becerra, M., Prado, S.D., Rodríguez-Belmonte, E., Cerdán, M.E., and Siso, M.I.G. 2002, Biotechnol. Lett., 24, 1391.
17. Holsinger, V.H., and Kligerman, A.E. 1991, Food Technology, January, 92.

18. Kumar, V., Ramakrishnan, S., Teeri, T.T., Knowles, J.K.C., and Hartley, B.S. 1992, Biotechnology, 10, 82.
19. Ramakrishnan, S., and Hartley, B.S. 1993, Appl. Environ. Microbiol., 59, 4230.
20. Domingues, L., Onnela, M.-L., Teixeira, J.A., Lima, N., and Penttilä, M. 2000, Appl. Microbiol. Biotechnol., 54, 97.
21. Domingues, L., Teixeira, J.A., Penttilä, M. and Lima, N. 2002, Appl. Microbiol. Biotechnol., 58, 645.
22. Domingues, L., Vicente, A.A., Lima, N., and Teixeira, J.A. 2000, Biotechnol. Bioprocess Eng., 5, 288.
23. Domingues, L. 2001, PhD Thesis, Universidade do Minho, Braga, Portugal.
24. Hodgson, J.A., Berry, D.R., and Johnston, J.R. 1985, J. Gen. Microbiol., 131, 3219.
25. Hammond, J.R.M. 1995, Yeast, 11, 1613.
26. Lima, N., Moreira, C., Teixeira, J.A. and Mota, M. 1995, Microbios, 81, 187.
27. Venâncio, A., Domingues, L. and Lima, N. 1999, J. Basic Microbiol., 1, 37.
28. Stratford, M. 1989. Yeast, 5, 487.
29. Domingues, L., Teixeira, J.A. and Lima, N. 1997, Biotechnol. Tech. 6, 399.
30. Domingues, L. 1997, MsC Thesis, Universidade do Minho, Braga, Portugal.
31. Mota, M. and Teixeira, J.A. 1990, Curr. Microbiol.,.20, 209.
32. Teixeira, J.A., Mota, M., and Goma, G. 1990, Bioprocess Eng., 5, 123.
33. Watari, J., Nomura, M., Sahara, H., and Koshino, S. 1994, J. Inst. Brew., 100, 73.
34. Lima, N., Teixeira, J.A. and Mota M. 1992, Bioprocess Eng., 7, 343.
35. Siso G.I.M. 1996, Bioresource Technol., 57, 1.
36. Domingues, L., Dantas, M.M., Lima, N., and Teixeira, J.A. 1999, Biotechnol. Bioeng., 64, 692.
37. Compagno, S., Porro, D., Smeraldi, C., and Ranzi, B.M. 1995, Appl. Microbiol.Biotechnol., 43, 822.
38. Domingues, L., Lima, N., and Teixeira, J.A. 2000, Biotechnol. Bioeng., 68, 584.
39. Domingues, L., Lima, N., and Teixeira, J.A. 2001, Biotechnol. Bioeng., 72, 507.
40. Lyons, T.P., and Cunningham, J.D. 1980, Am. Dairy Rev. 42, 42A.
41. Mawson, A.J. 1987, Aust. J. Biotechnol., 1, 64.
42. Gawel, J., and Kosikowski, F.V. 1978, J. Food Sci., 43, 1717.
43. Janssens, J.H., Bernard. A., and Bailey, R.B. 1984, Biotechnol. Bioeng., 26, 1.
44. Hahn-Hägerdal, B. 1985, Biotechnol. Bioeng., 27, 914.
45. Terrel, S.L., Bernard, A., and Bailey, R.B. 1984. Appl. Environ. Microbiol., 48, 577.