An integrated approach for cheese whey lactose valorisation

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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 fluctuating cells. Substrates 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.

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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 whey [2]. Lactose fermentation is certainly one of the alternatives. Lactose-containing whey streams could be used as abundant and renewable raw material for whey

![Diagram of whey processing](image)

Figure 1. Main process units for cheese whey valorisation.
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 valuable 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 high productivity continuous processes is implemented. High cell density continuous systems, keeping high cellular concentrations inside the bioreactor, are known for their high productivity. Among these, the ones that use flocculent cells are surely attractive due to their 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 metabolism potential candidates for cloning in S. cerevisiae cells. The bacteria Escherichia coli was one of the first microorganisms to be studied in respect to lactase genetics. Three different approaches can be utilised to express the E. coli  lacZ gene in S. cerevisiae. As β-galactosidase is cytosolic, lactose has to be transported to the cytoplasm in order to occur its hydrolysis. As a consequence, the lacF 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 lacE operon in S. cerevisiae in a multipurpose plasmid it was not possible to obtain transformants able of utilizing lactose [3]. The yeast transformants over expressing β-galactosidase were not able to grow on lactose due to the non-functionality 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 Sec61 [41] and Tam2 [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 membrane protein Gap1, it was possible to direct the E. coli β-galactosidase to the extracellular medium [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 utilisation 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 efficient, 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 [74]. More recently, the construction of recombinant S. cerevisiae strains secreting Kluyveromyces β-galactosidase has been reported [17, 16]. For that, the lacZ 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 hydrolyze 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;
- ease 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;
- minimized 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 the 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 their high productivity. Among these, the ones that use flocculent cells are surely attractive due to its simplicity and low cost.

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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 auxotrophic mutants and transformation of flocculent yeast cells

One of the prerequisites 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 auxotrophic mutants from a respiratory competent and highly flocculent S. cerevisiaeNCYC669strain [26]. The auxotrophic mutants were selected by growth on minimal medium with glycerol as carbon source supplemented with uracil and leucine, 5-fluoro-orotic acid (5-FOA). The mutant A3 was selected as it showed the best back mutation frequency (<6.5×10⁻⁶) and produced the same degree of flocculation. The A3 mutant was affected in the gene either coding for orotidine-5-phosphate decarboxylase (Ura2) or for orotidine-5-phosphoribosyltransferase (Ura3).

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 yeast cells was not affected by the uptake of foreign DNA [27]. Moreover, lithium ions are one of the ions known to have an inhibitory effect on yeast flocculation. Lithium ions are less pores 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 NCYC669-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. cerevisiaeNCYC669-A3 was used in a transformation procedure using plasmid pYES2-1 [11] together with a linear fragment from plasmid pYAC15 [13]. The plasmid pYES2-1 harbored 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 metabolization in the recombinant S. cerevisiae strain (Figure 2). The linear YAC4 fragment harbored the URA3 gene, used as a selective marker for recombinants. The selective medium used was 5-lactose-4-chloro-3-indolyl-β-D-galactoside (Xgal)-VNB-galactose. In this medium, only the clones that have the URA3 gene incorporated are able to grow. On the other hand, not all ara 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 Lac+ clones and the identification for positive Lac+ transformants. From a total of 1312 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 transformation were thereafter kept in
Figure 2. Illustration of the construction of the flocculent strain S. cerevisiae NCYC669-A3/T1.

VNIR/lactose medium, this 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 \( S.\ \text{cerevisiae} \) cell, the T1 transformant also presented bizarre forms and pseudo-yeastium and for that reason was not considered for further studies. The location of pKR1-B-Lac-4-1, as a plasmid in the recombinant strain was confirmed after restriction digestion analysis of the original pKR1-B-Lac-4-1 and plasmids isolated from the recombinant strain T1. Also the low stability of these transformants in non-selective medium indicated the presence of the pKR1-B-Lac-4-1 in its autonomous replicating form.

Figure 3. Phenotypic response of T1 and T2 transformants to the selective medium YNB-Xgal-lactose, control for \( S.\ \text{cerevisiae} \) (8c) and \( S.\ \text{carlsbergensis} \) (Kar7) (56).

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 \( S.\ \text{cerevisiae} \) NCYC669-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. Supposing this, the transformation of the same host strain, the mutant A3, with a plasmid harbouring an extracellular \( \alpha \)-agar [2-
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 metabolize 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 flocculated 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 inserted
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 L. nigro δ-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 L. nigro δ-galactosidase in yeast, the β-galactosidase
encoding gene lacA was coupled to the ADH1 promoter 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, W234, and the highly flocculent derivative
strain, W204-FLO1LNT1 [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](attachment:image.png)

Figure 4. Illustration of the constructions of the two brewer’s yeast strain (the non-flocculent strain W234
and the flocculent derivative W204-FLO1LNT1) and flocculent strain S. cerevisiae NCTC 6530.
AphVX11.)
observed with the recombinant strain *Saccharomyces 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 strain. 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-floculent W204-LE1 and the flocculent W204/TLO1(INT)/pL101, 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 maximized.

Using the dominant marker CUP1 allowed for the transformation of industrial strains. The recombinant strain 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 10 g/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-floculent 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 flocculast yeast cells for extracellular protein production, improved recombinant flocculent *S. cerevisiae* strains secreting β-galactosidase was constructed.

The flocculent *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 10 g/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 metabolized 30 g/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 SS1actose 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 6,235U 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 NCV869-A3pVK1.JJ 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 experiment was treated with an ultrafiltration membrane (Cerexiva-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).](image-url)
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 in β-galactosidase). In cheese whey, the main component responsible for the high BOD (Biological 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 NCYC609-A3/F1. 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 0.45g/Lh⁻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 [31]. 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 at 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 semi-cylindrical 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 11g/Lh⁻¹ was obtained (with a feed lactose concentration of 50g/L and a dilution rate of 0.55h⁻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]. Metabolic stability was also monitored throughout the continuous operation and found to be very similar to cell viability [36].

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

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<tr>
<th>Ethanol productivity (g/L h⁻¹)</th>
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<tr>
<td>0.15</td>
<td>[16]</td>
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<tr>
<td>0.19</td>
<td>[19]</td>
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<tr>
<td>0.2</td>
<td>[19]</td>
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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 a monthly operation this system proved to be stable with total conversion of lactose and constant ethanol conversion yield [26].

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 nonselective contaminants (due to continuous selection for flocculent cells). The high-cell-density continuous bioreactor operating with the recombinant flocculent strain S. cerevisiaeNCYC869-A/3F1 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 nonflocculated 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 pGL0 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 1x10⁷ 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, bacteri contamination is not a problem for these bioreactors [38].

3.2. Ethanol production from cheese whey permeate

Batch experiments showed that the recombinant S. cerevisiaeNCYC869-A/3F1 strain was capable of utilizing cheese whey permeate as a carbon and energy source, fermenting 50gL⁻¹ or 100gL⁻¹ of lactose present in cheese whey permeate without any additional nutrient supply [39]. Moreover, the complete lactose metabolism was observed in less than 48 hours, producing ethanol at 21gL⁻¹ and 53gL⁻¹ for initial lactose concentration of 50gL⁻¹ and 100gL⁻¹, 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 50gL⁻¹ total lactose consumption was observed with a conversion yield of ethanol close to the expected theoretical value. Using a continuously operating 5.5L bioreactor, ethanol productivity near 10gL⁻¹h⁻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 [41]. 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 (100gL⁻¹ lactose) flocculation was strongly impacted, and it became impossible to reach the dilution rates obtained with semi-synthetic medium with 100gL⁻¹ at deflocculated biomass was wasted 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 zein synthetic 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 in literature are presented. It is clear that the developed continuous high-cell-density system is very interesting as an ethanol productivity of near 10gl−1h−1 was obtained. This is more evident as there is still space for further development, once defoeculation has occurred when operating with concentrated cheese whey. If the concentrated cheese whey permeate is demineralized higher ethanol productivity can be expected.

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

<table>
<thead>
<tr>
<th>Organism</th>
<th>Bioreactor operation type</th>
<th>Substrate</th>
<th>Ethanol productivity (gl−1h−1)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kluyveromyces fragilis</td>
<td>Optimized batch process</td>
<td>Demineralized concentrated cheese whey permeate (CCWP) &gt;24gl−1lactose</td>
<td>5.2</td>
<td>[42]</td>
</tr>
<tr>
<td>K. fragilis</td>
<td>Continuous process with cell recycling, D=0.13h−1</td>
<td>CCWP + 100gl−1lactose</td>
<td>7.1</td>
<td>[43]</td>
</tr>
<tr>
<td>K. fragilis</td>
<td>Immobilized continuous process</td>
<td>Cheese whey</td>
<td>1.1</td>
<td>[44]</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>S. cerevisiae cells coimmobilized with β-galactosidase</td>
<td>Choice whey</td>
<td>4.5</td>
<td>[45]</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>Continuous operation with cell recycling, D=0.2h−1</td>
<td>CCWP + 55gl−1lactose</td>
<td>13.6</td>
<td>[46]</td>
</tr>
<tr>
<td>Recombinant S. cerevisiae</td>
<td>Continuous high-cell-density operation with flocculent cells</td>
<td>Whey permeate + 50gl−1lactose</td>
<td>8.0</td>
<td>[49]</td>
</tr>
</tbody>
</table>

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/pVR1.1. Experiments were done with lactose feed concentration of 20g/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) [21]. The best result for β-galactosidase productivity - 6.2×10^5 U.L^-1.h^-1 - was obtained for the system operating at 0.24 h^-1 dilution rate and for a 50g/L feed lactose concentration. Together with extracellular β-galactosidase production an ethanol productivity of 0.9g.L^-1.h^-1 was obtained for the bioreactor fed with 5g.L^-1 initial lactose concentration at 0.45h^-1 dilution rate.

The developed system is particularly attractive for applications 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 continuous 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