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Applications of Yeast Flocculation in Biotechnological Processes

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Abstract A review on the main aspects associated with yeast flocculation and its application in biotechnological processes is presented. This subject is addressed following three main aspects – the basics of yeast flocculation, the development of "new" flocculating yeast strains and bioreactor development. In what concerns the basics of yeast flocculation, the state of the art on the most relevant aspects of mechanism, physiology and genetics of yeast flocculation is reported. The construction of flocculating yeast strains includes not only the recombinant constitutive flocculent brewer's yeast, but also recombinant flocculent yeast for lactose metabolisation and ethanol production. Furthermore, recent work on the heterologous β-galactosidase production using a recombinant flocculent *Saccharomyces cerevisiae* is considered. As bioreactors using flocculating yeast cells have particular properties, mainly associated with a high solid phase hold-up, a section dedicated to its operation is presented. Aspects such as bioreactor productivity and culture stability as well as bioreactor hydrodynamics and mass transfer properties of flocculating cell cultures are considered. Finally, the paper concludes describing some of the applications of high cell density flocculation bioreactors and discussing potential new uses of these systems.

Keywords: recombinant yeast, flocculation, airlift bioreactor, heterologous protein production, fermentation

INTRODUCTION

Yeasts are, without any doubt, the most exploited microorganisms known and *S. cerevisiae* strains, the most representative yeast genus, are involved in the production of some important products consumed by human race such as bread, wine, beer, and distilled drinks. Fuel ethanol production using yeast is also of relevance.

Productivity increase is one of the main goals in any biotechnological process. This can be achieved in several ways:

- using new or modified strains;
- developing new bioreactors and optimising the operation strategies;
- improving the efficiency of separation processes;
- using efficient control systems.

Techniques that make use of cell immobilisation are clearly a very promising alternative as a way of improving biotechnological process productivity. In fact, the use of these systems presents several advantages:

- 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.

Among the several available immobilisation techniques, the use of flocculating microorganisms, due to its simplicity and low cost, is very attractive – no complex mechanical devices are needed as well as no support. This is a clear advantage over other immobilisation techniques since it is well known that support represents a major cost in immobilisation procedures.

However, at industrial scale, systems using flocculating yeast cells have been only utilised in the latter phase of primary beer fermentation to separate biomass from the fermented broth. No continuous fermentation systems using flocculating yeast cells have been implemented industrially, their implementation requiring a complete comprehension of the mechanisms involved in yeast flocculation and its control, in order to develop yeast strains with the adequate flocculation ability. They must be coupled with the appropriate bioreactor design and operating conditions.

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BASICS OF YEAST FLOCCULATION

Yeast Flocculation Mechanism

Yeast flocculation is defined as the non-sexual cell ag-

gregation (Fig. 1), which allows cell separation from the fermented broth. It can be inhibited by EDTA or sugars, and restored by Ca^{2+} ions. The observation of this phenomenon dates back to Pasteur scientific notes and it has been widely used in the brewing industry, probably for millennia. However, its mechanism has raised much controversy in this century. Early reports included sexual agglutination and chain formation in yeast flocculation [1]. Nevertheless, the mechanism involved in these three types of cell adhesion is different. In sexual agglutination, complementary haploid strains, α and a in case of *Saccharomyces cerevisiae*, exchange small peptide pheromones, α- and a-factors that cause a number of physiological changes. After these modifications, cells aggregate before nucleus-fusion to form diploids. Adhesion between cells is by protein/protein bonding between α- and a-agglutinins anchored in the complementary cell walls. Chain formation occurs when the bud cell fails to separate from the mother cell during yeast growth, resulting in chain formation as both the mother and daughter cells continue to form new buds. In this case the cells are physically joined at their cell walls. Chain formation is known to occur in *S. cerevisiae* strains due to nutrient deprivation or yeast strain mutation. Mill [2] reported formally the first proposed theory for yeast flocculation mechanism besides the colloidal theory based on surface charge neutralization (see for review [3]) that would not hold for Ca^{2+} specificity. Mill's theory stated that flocculated cells are linked by salt bridges with Ca^{2+} ions joining two carboxyl, phosphate and/or sulphate groups at the surface of two cells; the structures thus formed are stabilised by hydrogen bonds between complementary carbohydrate hydroxyl groups at the cell wall surface. The observed effect of pH on flocculation suggested carboxyl groups as the most likely combining sites [2]. Both carboxyl and phosphate groups can be considered as functional groups of amino acid side chains of the proteins on the cell walls, corroborating the loss of flocculation observed after treatment with proteases and protein-denaturing agents [4-11]. However, the calcium bridge hypothesis could not explain the observed inhibition of flocculation by sugars. Amri and collaborators [12] after a study on flocculent cell walls concluded that carboxyl groups were involved in flocculation but they also stated that the phenomenon of flocculation seemed more complex than the simple formation of a Ca^{2+} bridge, and that the involvement of "lectin like" components easily removed from the cell walls, should not be rejected. In 1982, Miki and collaborators [13] presented evidence for a new flocculation model stating that flocculation interactions may be mediated by a specific cell surface recognition mechanism, involving lectin-like binding of surface proteins to polysaccharides on adjacent cells. This model corroborates the calcium ions stereospecificity found in flocculation phenomenon and the sugar involvement [14] not explained by the bridge theory. In the lectin hypothesis the role of Ca^{2+} ions would be to maintain lectins in an active conformation. According to the lectin-like hypothesis, flocculation is mediated by the interaction between two distinct components of the same cell surface. The receptors, found both on flocculent and non-flocculent cells, are most probably α-branched mannans, as suggested by 1) mannose specific sugar inhibition [14] in *S. cerevisiae*, 2) lack of coflocculation with yeast known to lack mannan in its cell wall, such as *Schizosaccharomyces pombe* [13] and 3) mannan blocking and chemical modification experiments [13,15]. The presence of carbohy-

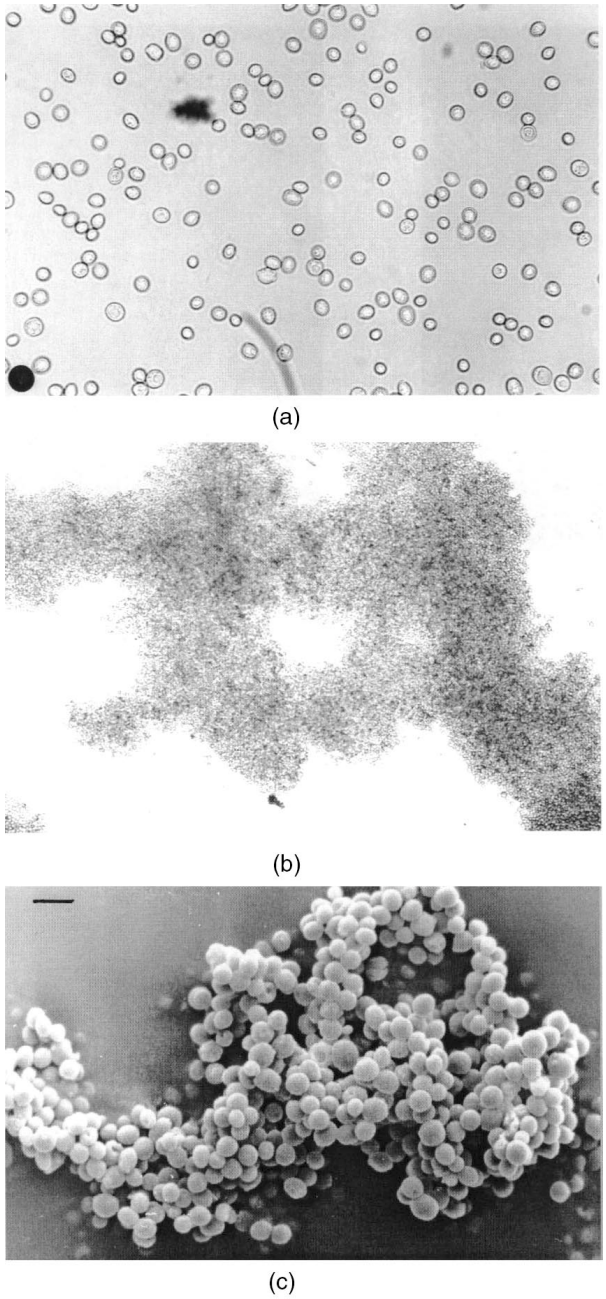

Fig. 1. Flocculent and non-flocculent *Saccharomyces cerevi-siae* cells photographs obtained by: (a) Non-flocculent cells (optical bright field microscopy 400X), (b) Flocculent cells (optical bright field microscopy 100X), (c) Flocculent cells (SEM; bar corresponds to 10 μm).

drate receptors was confirmed using known *mnn* mutants [16], which varied in wall mannan structure and showed that flocculation receptors were the outer chain N-linked mannan side branches, two or three mannose (Man) residues in length. For *Kluyveromyces lactis*, the structure of the phosphopeptidomannans from flocculent and non-flocculent yeast demonstrated that flocculent strains have a non-reducing terminal Man(α1,3)Man sequence [17], unlike non-flocculent strains. The receptors may be specifically bound to a lectin-like cognor, active only on the surfaces of flocculent yeasts.

Due to the protein lectin nature, attempts to isolate proteins unique to flocculation have been going on for many years. A number of possible protein bands on SDS gels have been found, ranging in size from 13 to 67 kDa [18] and antibodies against proteins on flocculent cells have been raised [19]. The presence of a 37 kDa protein in extracts of flocculent cultures was reported for *S. cerevisiae* strains [20], *K. marxianus* strains [11, 21,22], and flocculent *Hansenula anomala* strains [23]. A polypeptide of 13 kDa was also identified in alkaline cell extracts of flocculent *S. cerevisae* [24,25], as well as a 25 kDa protein [26]. In the *K. marxianus* case, the involvement of p37 in flocculation was recently supported by the fact that a *K. marxianus* mutant, deficient in the synthesis of p37, was no longer able to flocculate [27]. The isolation of surface lectin-like proteins from flocculent cells has been reported [28-32].

After careful examination of the physiology of a large number of laboratory and brewery flocculent strains, Stratford presented evidence of two groupings of yeast strains suggesting two different lectin mechanisms [33,34]. One group was termed Flo1 phenotype since it contained all strains bearing *FLO1* gene and also all other genes known to be involved in flocculation. This lectin mechanism was manno-specific. The other group was named the NewFlo phenotype, involving a large majority of brewery ale strains with unknown genotype, showing a manno- and gluco-specific lectin. The two phenotypes were distinguished by sugar, salt and low pH inhibitions, protease sensitivity, and selective expression of flocculation.

Although mainly studied in brewery yeasts, flocculation is spread over other yeast genera and bacteria. The mechanism of flocculation in other yeasts seems to follow the lectin theory (as supported by the data on isolation of proteins involved in flocculation) even though the lectin specificity could be different. For instance, galactose and its derivatives inhibit flocculation of *Kluyveromyces bulgaricus* and *Kluyveromyces lactis* species, as the interaction involves a galactose-specific lectin [28,29,35,36]. Also a study on the flocculation mechanism of a *K. marxianus* strain, as compared with a *S. cerevisiae* strain [37] suggested that the structure and/or spatial arrangement of the cell wall groups involved in flocculation were not the same in *K. marxianus* and *S. cerevisiae*, being nevertheless compatible with the lectin hypothesis.

The phenomenon of bacterial-induced yeast flocculation [38] seems to involve lectins from bacterial walls or, alternatively, the high molecular weight polysaccharides could be bridging between lectins on flocculent cells.

The adhesion of *Candida* species to cells of the host organism and many bacterial infections are mediated by surface lectins [39]. Curiously, lectins of infectious microorganisms are associated with surface structures termed fimbriae; similar structures have also been associated with flocculent yeast cells [40,41].

Considering the accumulated knowledge from flocculation genetics, a slightly modified lectin model was proposed [42], gaining support from subsequent experimental data. These authors proposed that flocculins might be (functioning as) lectins or at least have the sugar-binding properties associated with lectins. The major difference with Miki's model is that flocculins, being cell-associated glycoproteins, directly bind the mannoproteins of neighbouring cells. The flocculin, therefore, fulfils the same role as the two proteins in the Miki model, combining anchoring and binding properties. These authors also stated that the flocculation mechanism is basically the same for the NewFlo and Flo1 flocculation phenotypes, being the differences quantitative rather than qualitative. As a third flocculation phenotype group has been described [43] in which flocculation is mannose-insensitive and independent of Ca^{2+} ions, in this case a flocculation mechanism different from the NewFlo and Flo1 phenotypes is suggested. The flocculation lectin might correspond to the product of the dominant flocculation genes, such as *FLO1* or related genes, which have been known for a long time to play a major role in flocculation (see genetics of flocculation section). The deduced amino acid sequence from the *FLO1* gene revealed a serine- and threonine-rich protein with the N- and C-terminal regions that are hydrophobic and contain a potential membrane-spanning region [44], suggesting that the Flo1 protein is an integral membrane protein and a cell wall component. Further evidence has shown that Flo1p was a true cell wall mannoprotein [45,46]. In agreement with this idea Bony and co-workers [47] showed that the availability of Flo proteins at the cell surface of yeasts is well correlated with the flocculation level, being its distribution at the cell surface dependent on the constraints of cell wall biogenesis. The study of the Flo1 protein indicated that the hydrophobic C-terminus, which is a putative GPI anchoring domain, is necessary for the anchoring of Flo1p in the cell wall [45] and that the N-terminal domain of the protein is responsible for sugar recognition [45,46]. Supporting Teunissen and Steensma model [42], Kobayashi *et al.* [46] found that modification of two regions was required to change the mannose-specific sugar recognition pattern of the Flo1 protein to the mannose/glucose-specific pattern.

While experimental data accumulate elucidating the complex molecular mechanism for yeast flocculation, its role in nature is still unclear. In *K. marxianus*, the protein p37 involved in yeast flocculation can be induced by two different methods involving stress-continuous fermentation [48] or thermal stress [22]. In

S. cerevisiae, induction of flocculation by nutrient limitation has been considered [49]. More recently, it has been reported the involvement of some flocculation genes in filamentous growth [50-52], which are induced by nitrogen starvation. All in all, it seems that flocculation may be a defence mechanism adopted by some yeast strains, in response to adverse factors to the yeast cell. The floc formation may generate nutritionally rich microenvironments by selective lysis [53]. Further work is needed to determine the role of flocculation in nature.

Physiology of Yeast Flocculation

In 1958, Eddy and Phil [1] pointed out that despite the fact that the particular properties involved in flocculation were sometimes viewed as of an extremely variable nature, there were some that unless exactly controlled might lead to a false impression of variability. These properties include medium chemical composition, in particular salt and sugar content, pH, temperature, aeration, and agitation.

Flocculation development is influenced by medium salt content. Although some controversial data is found in literature concerning other cations, it is widely accepted the "activation" of flocculation by calcium ions. The presence of calcium ions is required at a very low concentration - 10^{-8} *M*, according to Taylor and Orton [54] - in order to induce flocculation. The controversial data involving other cations is due to the use of different cation concentrations, different flocculation measurement techniques, different pH values and strains with different genetical background. For instance, the *FLO5* and *FLO1* strains showed different patterns for the competing effects of other cations with Ca^{2+} [55]. For low salt concentrations (cations other than Ca^{2+}), there is an observed flocculation enhancement, while at high concentrations inhibition by salt is observed. The Ca^{2+} ion leakage or release from the cell promoted by other cations, namely magnesium ions, is one of the possible reasons for the flocculation induction at low salt concentrations [33]. In fact, Nishihara *et al*. [6] reported the need of the cation Mg^{2+}, at a minimal concentration of 20 μM, for flocculation to occur. Stewart and Goring [56] reported that Mg^{2+} and Mn^{2+} could imitate Ca^{2+} even though the flocculation intensity was lowered. The same authors reported that low concentrations of sodium and potassium (1-10 mg/L) also would induce flocculation on the strains studied. Other reasons for the promoting flocculation effect of low salt concentration are the lowering of yeast surface charge and the modifying effect on surface proteins, in a manner similar to that described as "salting in and salting out" for protein solubility [39]. Many salts are reported to cause inhibition of flocculation, depending on the concentration. These include the alkaline-earth metal ions Sr^{2+} and Ba^{2+} [10,33,54,55], Na^{+} [2,10,33,40,54], K^{+} [33,40,57], citrate ions [33], Ca^{2+} [58], Mg^{2+} [58], Mn^{2+} [55], Cs salts [58], Al^{3+} [55], La^{3+} [55] and Li^{+} [33].

Another important constituent in medium composition is sugar. Different sugars cause different effects on flocculation. Kihn *et al*. [59] reported that while one brewer's yeast strain (*S. cerevisiae* MUCL28323, top fermenting strain) was inhibited by mannose and other two (*S. uvarum* MUCL28235 and M259, bottom fermenting strains) were inhibited by mannose, maltose and glucose, there was no observable sugar inhibition in the top fermenting strains *S. cerevisiae* MUCL28733 and MUCL28734. Stratford and Assinder [34] grouped the flocculent yeast strains in two groups: those strains showing mannose sensitive flocculation (Flo1 phenotype), and those showing flocculation inhibition by several sugars (NewFlo phenotype). Later, Masy *et al*. [43] reported the existence of a third group, showing no flocculation inhibition by mannose or glucose. The sugar inhibition of flocculation is therefore strain dependent, having a direct effect rather than acting metabolically [34]. In *Schizosaccharomyces pombe* the flocculation inhibition by the sugar galactose and to a less extent, by glucose, has been reported, while for the sugar fructose and mannose, and for L(-) malic acid no flocculation inhibition was observed [60].

pH was first considered not to be a determinant factor of flocculation, being its effect mainly due to alteration of cell surface charge [39]. However, yeast flocculation is associated with different phenotypes, like the already mentioned Flo1 and NewFlo, which differ also in their pH tolerance. The Flo1 phenotype shows a very broad tolerance, exhibiting flocculation between pH 1.5 and 10 [39,61]. The NewFlo strains exhibited two distinct phenotypes: some flocculated over a broad range while others within a narrow pH range [61]. For the latter strains, pH is a determinant factor for flocculation to occur. Stratford [61] has shown that many brewing strains do not flocculate in laboratory culture media, because their initial pH and buffering capacity do not allow for the pH range within which these yeasts flocculate. Once the pH was corrected the brewing yeast strains were able to flocculate in laboratory culture media. With these strains, a simple change of pH at any desired time during fermentation, allows for cell separation from the medium [61]. Also, Yang and Choi [62] found for a *S. cerevisiae* strain that flocculation was induced by pH upshift.

Temperature may influence flocculation development and expression. There is an apparent contradiction in the literature; some authors noted deflocculation with increasing temperature while others noted an increase in flocculation with increasing temperature. This discrepancy may be attributed to differences in the response of ale and lager strains (reviewed in [63]). Nevertheless, it was shown that temperature did not inhibit cell-cell interactions, but it seemed to induce or repress the formation of a cell wall component involved in flocculation. As the yeast secretory pathway is prone to temperature-sensitive mutations [39], the temperature may alter the availability of the Flo protein rather than having a direct effect on flocculation. For *Kluyveromyces marxianus* strain, flocculation can be induced by a temperature upshift [22]. Once more, this effect was related to an increase in the synthesis and subsequent accumu-

lation of a protein in the cell wall.

The aeration effect on flocculation seems to be related with the mitochondrial function. The presence of respiratory inhibitors represses the flocculation induction [6] and the respiratory-deficient mutants show different flocculation behaviour when compared to parental strains [20]. The induction of flocculence is repressed by cycloheximide, but not by chloramphenicol. Nishihara *et al*. [6] concluded that mitochondrial function was more important than synthesis of mitochondrial proteins. As mitochondrion affects the secretion process, the mitochondria effect on flocculation can be also an indirect effect [39]. Another indirect effect considered in flocculation is the oxygen influence by increasing the cell wall hydrophobicity [64], which has been related with flocculation by several authors [23,49,64-69]. The combined effect of aeration and glucose concentration has also been studied [70,71], revealing that for extreme aeration conditions (0.1 and 5 vvm) the initial glucose concentration was not important while for an intermediate aeration rate of 1 vvm, high glucose concentrations (6 to 10% w/v) increased flocculation [71].

Agitation has two antagonistic effects: enhanced particle collision rate induces flocculation on the one hand, and on the other higher shear forces cause particle breakage [72-74]. An increase in agitation intensity leads to a decrease in floc size [75,76]. Being so, gentle agitation gives large flocs while vigorous agitation gives smaller denser flocs that settle more slowly but give a compact sediment [74-76].

The environmental properties mentioned above may be controlled allowing not only for a better understanding of the phenomenon but also for its control. Nevertheless, the strain variability must be taken into account, as this is one of the reasons for most of the controversy found in literature data concerning flocculation.

Genetics of Yeast Flocculation

Due to the relevance of flocculation on the brewing industry, much effort has been given to elucidate the genetic and environmental control of flocculation. The control of flocculation is sought not only to be applicable in the brewing industry, but also to make practical application of the phenomenon in biotechnology.

During the early 1950's Gilliland [77] and Thorne [78] independently carried out a study on the yeast flocculation genetics, establishing the existence of flocculation genes by genetic crosses. Until the 70's, flocculation was recognized to be dominant and to be under the control of multiple gene pairs [79]. Despite the difficulties concerning tetrad analysis of brewing strains due to their low spore viability, in the 70's several genes conferring flocculation were recognized. Lewis and Johnston identified the dominant flocculation genes, *FLO1* [80] and *FLO2* [81] and the recessive gene *flo3* [82]. Stewart and collaborators identified the dominant gene *FLO4*, located in chromosome I [20,83]. Further studies indicated that *FLO1*, *FLO2* and *FLO4* are in fact allelic [84]. The identification of chromosomal genes responsible for flocculation lead to some enthusiasm, but further studies revealed that flocculation is not a straightforward molecular mechanism. According to our present knowledge, at least 35 genes were identified as to be involved in flocculation (reviewed in [42]). A gene family consisting of two dominant genes discovered by classical genetics, *FLO1* and *FLO5*, and the two dominant genes, *FLO9* and *FLO10*, identified on the basis of sequence homology to *FLO1* [42] encode for proteins with a high degree of homology. The dominant *FLO11* gene, with a high degree of homology with the *STA1* gene, is more distantly related to this gene family, with a similarity of 37% to the product of the *FLO1* gene [85]. The *FLO9* gene product is 96% similar to the *FLO1* product; *FLO10* product is 58%, while the *FLO1* and *FLO5* gene products are 96 % similar [42]. Recently, a new *FLO* gene sharing no homology with the *FLO1* gene has been described and mapped on the right arm of chromosome XII [86]. The new gene confers a strong flocculation phenotype, comparable in all aspects to that induced by *FLO1*, and was named *FLO2* [86]. The transformation of a *flo1* mutant with pSV1 vector carrying *FLO2* gene resulted in a flocculation phenotype. The authors [86] suggested that *FLO2* could be a structural gene, similar in function (but not in sequence) to *FLO1* that would normally be silent in all *S. cerevisiae* strains but otherwise active when cloned in a plasmid. From Southern analysis and data from the *Saccharomyces* genome database it has been shown that *FLO2* activates silent *FLO* genes (other than *FLO1*) in non-flocculating strains [86]. Further work is required to elucidate the *FLO2* function. Some controversial data has involved the dominant *FLO8* gene [87-93]. Finally, Kobayashi and collaborators [94] cloned the *FLO8* gene and their results suggested that the *FLO8* gene mediates flocculation via transcriptional activation of the *FLO1* gene. These authors also suggest that the *FLO8* gene is located on chromosome V and not VIII as reported by Yamashita and Fukui [88]. The *FLO5* gene has been first mapped on chromosome I by cytoduction [95] and its localization was confirmed later by cloning the *FLO5* gene [96]. The semi-dominant and recessive genes *flo3*, *flo6* and *flo7* are probably allelic to one of the flocculation genes *FLO1*, *FLO5*, *FLO9* and *FLO10* [42]. Besides the four dominant genes (*FLO1*, *FLO5*, *FLO9* and *FLO10*), the transcriptional regulatory gene *FLO8*, the new *FLO2* gene, the semi-dominant *flo3* and the recessive *flo6* and *flo7*, mutations in several genes have been found to cause flocculation (for details see review in [42]). Some of these mutations involve regulatory genes, like the *TUP1* and *SSN6* genes, others mitochondrial genes like the *oli1* and *oxi2* [97], or genes involved in cell wall biosynthesis. The expression in yeast of heterologous genes like the human *Ha-ras* and the viral *tax* gene caused yeast flocculation. The mechanism behind the flocculation induction in such different situations is far from being understood. More recently, the overexpression of the *GTS1* gene has been reported to result in

constitutive flocculation [98], even when expressed in a yeast strain lacking the *FLO1* gene, suggesting that the flocculation mediated by these two genes is unlinked. Overexpression of *GTS1* results in an increase of thermotolerance (broadening the tempera-ture growth optimum and increasing lethal heat shock resistance) and in a change in the cell wall long-chain fatty acids profile (leading to a reduced cell surface charge and increased hydrophobicity). Further work is needed to elucidate the mechanism underlying the constitutive flocculation effect of *GTS1* gene overexpression.

As mentioned before, the four dominant structural genes, *FLO1*, *FLO5*, *FLO9* and *FLO10*, encode for highly homologous proteins. These proteins are anchored in the cell wall with the C-terminus, while the N-terminal sugar-binding part is protruding in the medium [42,44-47]. All strains containing these *FLO* genes belong to the Flo1 phenotype [34]. Until recently, nothing was known about the genetics of the NewFlo phenotype flocculation. Kobayashi and collaborators [46], have isolated a new *FLO1* homologue, Lg-*FLO1*, from a bottom fermenting yeast strain belonging to the NewFlo phenotype, and replaced the *FLO1* gene in a Flo1 phenotype strain with the Lg-*FLO1* gene. They showed that the *FLO1* and Lg-*FLO1* genes encode a mannose-specific and mannose/glucose-specific lectin-like protein, respectively, identifying the region responsible for sugar recognition. These data support the slightly modified lectin model proposed by Teunissen and Steensma [42], being the flocculation mechanism basically the same for both the NewFlo and Flo1 flocculation phenotypes. However, a third group named mannose-insensitive has been described by Masy *et al*. [43] which is insensitive to mannose and independent of Ca^{2+} ions. They [43] suggested that flocculation in such strains could be produced by hydrophobic interactions or other specific interactions not involving mannans. It is likely that the flocculation mechanism of these strains is different from the Flo1 and NewFlo mechanism. The *GTS1* overexpression could be related to this type of flocculation since an increase in hydrophobicity has been noticed [98]. However, further data are required.

Recently, the overexpression of the *Kluyveromyces marxianus GAP1* gene, encoding for the p37 protein, in *Saccharomyces cerevisiae*, resulted in flocculation of the transformed yeast strain [99]. The transformed strain exhibited a flocculation/deflocculation phenotype very similar to that of *K. marxianus*. Again, other mechanism rather than the Flo1/NewFlo flocculation mechanism might be present. Interestingly, the cell wall hydrophobicity correlates linearly with the flocculation ability of this strain, being the major determinant in the flocculation ability of *K. marxianus* [68]. It is worth noting that being hydrophobicity a function of cell wall protein and composition [100], the influence of hydrophobicity in flocculation does not rule out the lectin theory.

DEVELOPMENT OF NEW FLOCCULENT YEAST STRAINS

Flocculation is a determinant yeast property in brewing being considered the chief factor in termination of fermentation [39]. If flocculation occurs early, removing suspended yeast, attenuation will cease and result in a hung fermentation containing residual sugar. The control of yeast flocculation is therefore of prime importance to the brewing industry. Moreover, the growing interest in flocculation bioreactors due to their high cell density and high productivity in continuous processes emphasizes the need for controlling yeast flocculation. The flocculent brewery yeasts, which only flocculate in the stationary phase of growth, are unsuitable for use in continuous bioreactors, since single exponentially growing cells would be washed out [39]. The advantages of flocculating systems led to research aiming not only at controlling flocculation, but also at inserting new properties into constitutively flocculent yeast strains for industrial applications.

Transfer of Flocculation Properties to Non-flocculent Yeasts

In 1980, Barney and collaborators [101] reported the genetic transformation of flocculence in *Saccharomyces cerevisiae*, without a vector, by the induced uptake of native yeast DNA by spheroplasted, nonflocculent recipients. They demonstrated the cotransformation of the linked *ade1* and *FLO1* gene, proving that the transfer of a gene for flocculence (*FLO1*) to a nonflocculent yeast, results in a new flocculent yeast strain.

Aiming at improving the yield of the batch process in cane molasses fermentation and using a flocculent yeast for a continuous molasses or cane juice fermentation, Figueroa *et al*. [102] reported the transfer of the flocculation property to the yeast *S. cerevisiae*. This yeast is used as distillery yeast in batch processes in Argentina for ethanol production from sugar cane molasses. Also for the improvement of biotechnological ethanol production, the construction of hybrids between a *S. cerevisiae* saké strain and a laboratory flocculent *S. cerevisiae* NCYC 869 strain (*FLO1*) by intraspecific protoplast fusion was reported [103]. The new hybrids contained both the fermentation characteristics of industrial *S. cerevisiae* saké strain and flocculence.

While *FLO1* gene expression is subjected to mating-type regulation, the expression of the *FLO5* gene is not affected by the mating-type locus, suggesting the potential usefulness of the *FLO5* gene in constructing polyploid flocculent strains [104]. The hybrid strains, constructed by sexual mating between a wine/brewer's yeast and a laboratory flocculent yeast strain (*FLO5*), showed strong flocculation ability [104].

The *FLO1* gene has been cloned, sequenced and transformed to both top- and bottom- fermenting non-flocculent yeasts [25,44,91,105]. As it was known that stability of the YEp-type plasmid was extremely high in bottom-strains under non-selective condition, the cloning of the *FLO1* gene was first performed by a multi-

copy plasmid [105]. In addition, the multi-copy of the *FLO1* gene may relieve the mating-type repression in industrial strains being useful in the construction of flocculent industrial strains [105]. Despite the fact that the modified strains were genetically unstable [105], no substantial difference was found between the trial and control beers. The same gene, *FLO1*, has been integrated in the genome of a non-flocculent bottom brewing yeast strain, resulting in a stable constitutive expression of the flocculation phenotype [106].

So far, the *FLO1* gene has only been expressed constitutively in transformed brewing yeasts. As early flocculence during fermentation should not occur because it significantly slows down the fermentation rate, by appropriate promoter constructions, the flocculation can be induced at a later stage of beer fermentation.

The introduction of flocculation property into wine yeasts (*S. cerevisiae*) by hybridisation has been reported [107]. More recently, the transfer of the flocculation property to a non-flocculent *S. cerevisiae* by cloning the heterologous gene *GAP1* from *K. marxianus* has been reported [99].

For the flocculent yeast strains constructed aiming at continuous biological ethanol production, the constitutive flocculation expression phenotype is desired. In this case, the stability of the flocculation characteristic is the main concern.

Transfer of Relevant Industrial Properties to Flocculent Yeast

Flocculent yeasts allow for the operation at high cell density, with overall increased productivity. As flocculent cells easily separate from the surrounding medium, its use is attractive even for batch or fed-batch operation (Fig. 2). The continuous operation at high cell density with flocculent cells has the same main advantages of immobilised systems, facilitating the downstream processing and presenting higher overall productivity. In addition, it is economically more attractive as there is no need for the support. Being so, the development of flocculent cells for ethanol production from cheap raw materials or even for protein production is considered to be an attractive bioprocess.

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 [108]. Recombinant DNA methods have already been used to introduce several new properties into brewing yeasts such as the ability to degrade carbohydrates, modifications to the processes responsible for beer flavour production, and the already referred changes to yeast flocculation [109]. However, it is worth noting that brewing yeasts flocculate only at the end of fermentation. Thus, they can be genetically modified under a non-flocculent state, which is not the case for constitutively flocculent yeast cells.

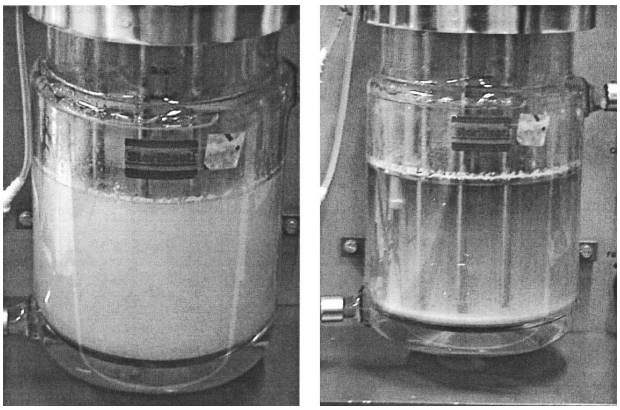

Fig. 2. View of a flocculating yeast cell culture with stirring (150 rpm.) (a) and 45 seconds after stirring was stopped (b).

UV radiation was used to construct *ura*$^-$ mutants from a respiratory competent and highly flocculent *S. cerevisiae* NCYC 869 strain [103]. The auxothrophic mutants were selected by growth on minimal medium with glycerol as carbon source, supplemented with uracil and 1 mg/mL 5-FOA. The mutant A3 was selected as it showed the best back mutation frequency ($<6.5\times10^{-10}$) and produced the same degree of flocculation. The A3 mutant was affected in the genes either coding for orotidine-5'-phosphate decarboxylase or for oritidine-5'-phosphate pyrophosphorylase. The mutation was confirmed to be *ura3* by complementation with a plasmid containing the *URA3* marker [110,111]. For the flocculent yeast transformation, the lithium acetate method was preferred. In contrast to spheroplast transformation method, which implies cell wall regeneration, the lithium acetate method avoids perturbation of cell walls. In fact, the flocculence ability of yeasts was not affected by the uptake of foreign DNA [111]. It is noteworthy that as the lithium ion inhibits flocculation [33], the cells are dispersed in the uptake DNA phase, and thus present transformation efficiencies similar to non-flocculent cells [111].

Aiming at producing ethanol from lactose-composed raw materials, a flocculent *S. cerevisiae* strain fermenting lactose was constructed [112]. The auxotrophic mutant A3 was used in a co-transformation procedure using the KR1B-Lac4-1 plasmid [113] together with a linear fragment form YAC4 [112]. The plasmid KR1B-Lac4-1 harboured the *LAC4* gene and *LAC12* gene while the linear YAC4 fragment harboured the *URA3* gene marker. The recombinant strain was able to grow and fermented the substrate lactose. Even though the recombinant strain was able to flocculate, it presented a different behaviour from the host strain. 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 cloning of the lactose permease, a cell membrane protein. Because flocculation is a cell wall phenomenon,

introduction 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 the plasmid pVK1.1 [114] harbouring an extracellular *A. niger* β-galactosidase, resulted in a recombinant flocculent *S. cerevisiae* strain with the same flocculation ability as the host strain [115]. Nevertheless, the flocculation instability should not be a problem as it can be overcome by a selective bioreactor operation for the flocculent cells [116-118]. The constructed recombinant strain is very attractive for industrial use in cheese whey bio-remediation. As the *S. cerevisiae* strain is not able to metabolise the sugar lactose, the only carbon source present in cheese whey, there is no need for extra selective pressure: once the recombinant strain looses the plasmid, it will not be able to grow.

Besides ethanol production, flocculent yeast cells can also be used for protein production by secretion. This was demonstrated by constructing two brewer's yeast strains, a flocculent and a non-flocculent, both secreting an *Aspergillus niger* β-galactosidase [115]. No significant differences between the β-galactosidase activity were detected in the supernatants from the non-flocculent and flocculent recombinant brewer's yeast strains. A slight β-galactosidase accumulation inside the floc was suggested but it seemed that the protein was able to diffuse through the porous floc afterwards [115]. Anyway this can be minimised by using polymeric additives that increase floc porosity [119,120]. Protein production using continuous high cell density flocculent systems will be therefore viable and economically attractive, especially when the protein is secreted. In such situation not only the protein separation process is simplified but also an increased overall volumetric productivity can be expected [115].

Other characteristics of industrial importance may be incorporated in flocculent microorganisms. Javadekar *et al.* [121] introduced the killer character in an industrial flocculent strain of *S. cerevisiae* (NCIM3528) by protoplast fusion with the *S. cerevisiae* NCIM3578, carrying the killer character.

The development of new flocculent yeast strains is the first step of process optimisation, being the following one the development of the appropriate bioreactor design and operation conditions.

BIOREACTORS FOR YEAST FLOCCULATION

Bioreactor Design and Operation Conditions

In continuous fermentation systems, the operation of bioreactors using flocculating organisms implies that the adequate size, shape and density characteristics of the flocs are achieved and maintained, in order to retain biomass inside the bioreactor as much as possible, while keeping the maximum activity. In other words, this means that a balance must be obtained between the capacity of the bioreactor to retain biomass (favoured by an increase in floc size) and the minimisation of the flocs mass transfer limitations (favoured by smaller flocs). This balance depends on the bioreactor hydrodynamic conditions (especially shear stress) and yeast cells flocculation ability and flocs physical properties. Stirred vessels and packed beds are therefore not recommended as flocculation bioreactors (although they may be suitable for other immobilised cell systems which are mechanically more resistant). The use of a fluidised bed for flocculating cultures is not advisable, either, as the density difference between flocs and medium is rather small and fluidisation would be achieved at very low air/liquid flow rates, enhancing the mass transfer limitations already existing. On the contrary, bubble columns and airlift reactors (especially the later) are being extensively used with three-phase systems in processes involving flocculating organisms [60,118,122-129].

The evaluation of the performance of flocculating cultures in airlift reactors depends on the understanding of the interaction between design and operational parameters [130].

As one of the main features of high cell density systems is the high hold-up of the solid phase (that may be as high as 50-60% of the total bioreactor volume, with the corresponding reduction in liquid phase volume) a significant research effort has been devoted to optimise the design of several parts of three-phase airlift reactors, namely in those aspects related to their use as high cell density systems. As previously stated the use of flocculent microorganisms requires a surplus of attention when designing a reactor in order to keep flocs with the suitable characteristics for the process (shape, density and size, mainly).

Influence of the Solid and Liquid Phases in High Cell Density Bioreactors Hydrodynamics

In order to increase biomass performance using yeast cell flocs, it is of crucial importance to characterise the properties of the solid phase (particularly solid phase hold-up) and liquid phase [131], and the way they affect the hydrodynamics of flocculation bioreactors. However, no real data are available for flocculation bioreactors and so far, even when systems with similar properties have been used as models, only a few of them deal with solids loads as high as those found in flocculation bioreactors. In fact, solids load may affect gas-liquid mass transfer efficiency [132], gas hold-up [133,134], liquid velocity [135], mixing time [128] and solids hold-up in the different parts of the bioreactor [134], among other parameters.

In flocculation bioreactors, biomass retention is strongly improved when an enlarged top section is used as it permits a better gas disengagement as well as enhanced solids settling [136]. In fact, when a full characterisation of solid phase distribution inside the bioreactor was done, for all tested solids loads and gas flow rates, solids hold-up in the separator has been found to be lower than that in the riser or in the downcomer, thus proving the efficiency of this system in what con-

cerns solids retention [137].

Mass Transfer in Flocculating Cell Cultures

Mass transfer limitations in high density flocculating yeast cell cultures are mainly associated with the transport of solutes (being either nutrients or metabolic products) inside the flocs and, although to a smaller extent, to the gas-liquid mass transfer of oxygen from the gas phase to the liquid medium. It is fundamental to quantify these resistances in order to characterise the process and to decide which measures must be taken to minimise their effects in the overall result of the process. Concerning the gas-liquid mass transfer of oxygen, and especially in the case of aerobic fermentations, cell exposure to low or near zero dissolved oxygen concentrations may have a deleterious effect on metabolism, therefore affecting the overall yield of the process. Due to the low solubility of oxygen in fermentation media, there is the need of a continuous O_2 supply, either pure or as part of a gaseous mixture (most frequently, air). The design parameters of an airlift as well as the presence of solids may affect gas-liquid oxygen transfer. In the case of the former, the presence of a draught tube and the flow area ratio between riser and downcomer [138] affect significantly the oxygen volumetric mass transfer coefficient (k_La). The presence of solids usually provokes a decrease of k_La, when compared to the value for two-phase operation [133,139,140], and the same happens in the particular case of the solids being yeast cell flocs [129].

Concerning the mass transfer of solutes inside the flocs, diffusion is the most important mechanism and it is generally described using a single parameter, the effective diffusivity (D_e) which relates the gradient of the characteristic concentration ($c(r,t)$) along the coordinate r at time t to the average diffusive solute flux (J_D) across the volume of the object in study, which is expressed by Fick's law:

$$J_D = -D_e \cdot \nabla c\ (r,t) \tag{1}$$

Very little work has been done for the characterisation of solutes transport inside flocs [141-143] and the existing data on the diffusivity of glucose and oxygen do not usually refer to the case of cell aggregates (biofilms not included) [144].

The assessment of internal diffusion limitations in yeast flocs was done by Teixeira and Mota [141], using a membrane bioreactor. A diffusion-reaction model was used and the ratio between the specific lactose uptake rates of a flocculating strain (*K. marxianus*) over a non-flocculating strain (*i.e.*, an effectiveness factor - η) was determined to be 0.50 and its value was related to the importance of mass transfer limitations inside the yeast flocs. The floc porosity was also determined by means of a thermogravimetric balance, obtaining a value of 50.5%. This value increased about 10% when the flocs were grown in the presence of a polymeric flocculation additive (a cationic resin) causing a corresponding 10% increase in the value of the effectiveness factor.

This was an example of many of the attempts that have been made in order to reduce diffusional limitations in flocs through the use of polymeric additives [142,145,146]. Those additives should enlarge the space between adjacent cells, extending the bridges that link the cells in a floc [119]. In fact, reductions in diffusional limitations have been reported suggesting an increase of the effective diffusion coefficients of the substrates in the floc, whenever some flocculating additives are used. Lima *et al*. [119] studied the influence of several polymeric additives on specific glucose uptake rate of flocs of *S. cerevisiae*, using the same membrane bioreactor of Teixeira and Mota [141]. An increase of glucose uptake rate by cells in the flocs grown in the presence of additive was always observed when compared to those grown without additive: 19% for bis [polyoxyethylene-bis(amine)] 20,000, more than 50% for BPA 1,000 and two-fold for Magna Floc LT25. Floc porosity was measured and found to range from 55.7% (without additive) to 60.5–63.0% (with additive). The authors proposed a model for the cell arrangement of yeast flocs, characterised by a cubic packing of the cells. This model succeeded in explaining both the increase in the available area for substrate flux inside the floc when a flocculation additive was used, and the consequent increase in the overall reaction rate. Sousa and Teixeira [142] studied the influence of an anionic (Magna Floc LT25) and a cationic polymer (BPA 1,000) on the batch fermentation parameters of the same flocculating *S. cerevisiae* strain. While the cationic polymer showed to have little effect on the kinetic performance of the system, the anionic polymer caused a two-fold decrease of the time needed to obtain total glucose consumption, confirming its positive effect on the reduction of mass transfer limitations inside flocs. Through the calculation of the effectiveness factor (η), the same authors [127] estimated the penetration depth of oxygen in the flocs, corresponding to fractions of cells in the floc having oxygen available ranging from 2.4% to 16.2%. This estimate was made considering that oxygen uptake by yeast follows zero-order kinetics.

The estimation of the penetration depth of the solutes in flocs was also done by using data on substrate diffusion inside flocs and modelling diffusion-reaction phenomena.

The direct measurement of diffusion coefficients in flocs is made difficult by their very fragile nature: in fact, flocs are easily destroyed and this problem becomes more acute with the size increase, namely when dealing with diameters between 2 mm and 3 mm. Further, floc dimensions and shape are of capital importance to the assessment of D_e [147] and their geometry is seldom a perfectly defined sphere, as it is usually assumed, being flat cylinders and ellipsoids the most common shapes. One of the best procedures to determine their diameter in a non-destructive way is by image analysis. Such a technique applied to yeast flocs has been developed by Vicente *et al*. [148], where both the floc size distribution of different populations and the

number of flocs present in the treated samples have been determined fitting a Gauss curve to the experimental data. From there, the values of the average floc size and their respective standard deviation can be calculated.

Libicki *et al*. [144] calculated the effective diffusivity of nitrous oxide, a non-reactive solute, within cell aggregates of *Escherichia coli*. Other authors used inactivated cells [143], measuring the oxygen transfer characteristics of aggregates of *Solanum aviculare* with 3 mm to 12.5 mm in diameter. Effective diffusivity of oxygen in deactivated aggregates was found to increase with particle diameter varying between 2% and 40% of the molecular diffusivity of oxygen in water at the same temperature. The authors therefore considered that severe oxygen limitations occurred in the aggregate. The same conclusion was drawn by Vicente *et al*. [149], who studied mass transfer characteristics (effective diffusivity, D_e, and external mass transfer coefficient, K_c) of glucose and oxygen in flocs (0.90 mm to 2.42 mm in diameter) of *S. cerevisiae* using inactivated cells but a different technique. A modified diffusion cell [150] was used in order to avoid floc destruction. Diffusion coefficients were found to be, for glucose, 17% of the diffusivity in water and, for oxygen, from 0.2% to 1% of the diffusivity in water, which is in agreement with the data from Ananta *et al*. [143], if the floc size is considered. K_c values increased with the agitation rate, as expected, and have values ranging from 7.5×10^{-9} m/s to 15×10^{-9} m/s. These values indicate that both the mass transfer inside and outside the flocs may be a limiting step in this process.

Based on these results, concentration profiles of glucose and oxygen inside aggregates of *S. cerevisiae* were simulated and calculations were made for different possible sizes of the yeast flocs, considering also the presence or the absence of a polymeric additive [151] (Fig. 3(a) and (b)).

From Fig. 3 it can be seen that only a small percentage of the cells in the floc metabolise glucose oxidatively, due to severe oxygen limitations. The presence of the polymeric additive, however, increased the ratio of cells operating under respiratory metabolism over those under fermentative metabolism: from 0.4% to 5.7%, without additive, to 1.2% to 8.5%, with additive, depending on the bulk glucose concentration.

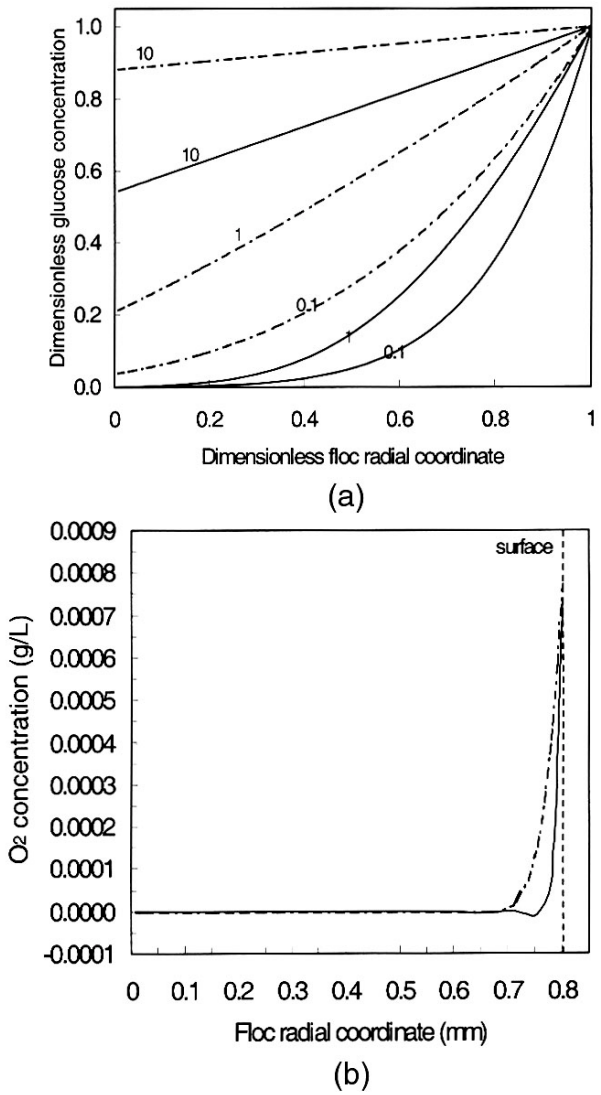

Fig. 3. Comparison of the concentration profiles for flocs grown with (dot-dashed lines) and without (solid lines) a flocculation additive, with a radius of 0.8 mm: (a) dimensionless glucose concentration profiles (the parameters near the lines identify the values of the bulk concentration to which each line corresponds), (b) dissolved oxygen concentration profiles.

Operation of Flocculation Bioreactors

One of the main advantages associated with the operation of yeast flocculation bioreactors is their operational stability and their resistance to contamination by other microorganisms, even those with higher specific growth rates, as it has been clearly demonstrated by Domingues *et al*. [152]. When operating with a recombinant *S. cerevisiae* strain [112] at a dilution rate of 0.45 h^{-1}, it was possible to significantly reduce an artificially introduced bacterial contamination within 4 h. Operation stability is patent in Fig. 4, where the operation of an external loop airlift with a sedimentation zone (a volume of 1.2 L and fed at different dilution rates) is shown [153].

This experiment was made with the yeast strain *K. marxianus ATCC 10002* that, when inoculated in the bioreactor, had no flocculation ability. Being so, during the first 12 days there was no considerable difference between the biomass concentration in the effluent and inside the bioreactor. In the following days, however, flocs became apparent and the biomass concentration started to rise inside the bioreactor, while the effluent biomass concentration was kept near zero. This means that both the hydrodynamic conditions inside the reactor were favourable to the formation of flocs and that there was a selection of the most flocculent individuals

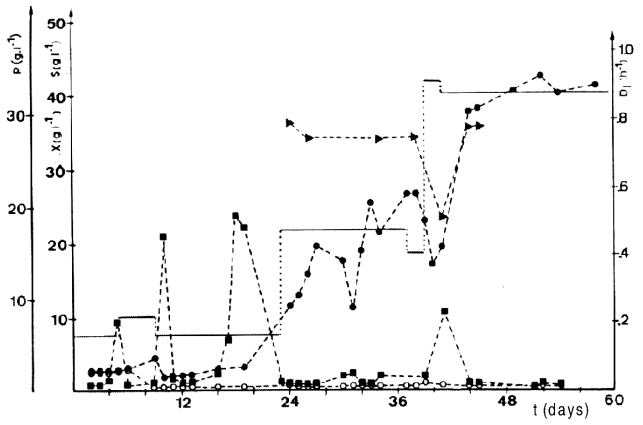

Fig. 4. Flocculation bioreactor response to increasing dilution rate (*D*) with a feed lactose concentration of 57.2 g/L; (●) biomass concentration in the bioreactor (*X*), (○) biomass concentration in the effluent (*X*), (■) lactose concentration (*S*), (▲) ethanol concentration (*P*).

in detriment of the non-flocculating ones, due to the sedimentation characteristics of the former. Of particular importance is the fact that it has been possible to keep constant the flocculating characteristics of the strain used during a working period of two years. It is interesting to notice the effects of a sudden increase of the dilution rate (in days 39 to 41), when a peak of lactose concentration together with a drop of both ethanol and biomass concentrations are registered and are essentially due to a washout effect. However, the system reacts and, three days later, a new equilibrium state is reached. Also noteworthy is the biomass concentration in the effluent, which has always very low values throughout the course of the experiment. With this system it has been possible to achieve a practically complete conversion of substrate during an alcoholic fermentation of lactose [117]. A maximum ethanol outlet concentration of 44.8 g/L and a maximum ethanol productivity of 24.4 $g \cdot L^{-1} \cdot h^{-1}$ were obtained.

Considering the need to develop new and simpler fermentation systems and the suitability of the airlift bioreactor for cultures using flocculating microorganisms, a 5.4 L internal loop airlift bioreactor was tested and compared with the previous system [126] using a highly flocculating strain of *S. cerevisiae* growing on glucose. A comparison was made in terms of start-up evolution, overall performance and power costs. The best ethanol productivity was obtained for the concentric tube airlift reactor (12.9 $g \cdot L^{-1} \cdot h^{-1}$), but both systems behaved in a similar way and the productivity values were about seven times higher than those in commercial systems. There was also a clear indication of a higher cell activity in the concentric tube airlift bioreactor when compared to the external loop airlift, thus compensating for the lower cell retention capacity of the former. The power cost analysis revealed differences only at laboratory and pilot scales; at industrial scale, however, the concentric tube airlift is advantageous because no mechanical parts are involved in cell recycling. The work proceeded, then, with the concentric tube airlift [127], by studying the evolution of fermentation parameters of the same flocculent strain of *S. cerevisiae* during the start-up of a continuous fermentation. A strong influence of the dilution and aeration rates was found on both biomass and ethanol concentrations and kinetic parameters. The operating parameters, in turn, do not seem to affect glucose consumption rates but affect, instead, the stoichiometry of its conversion to either biomass or ethanol, suggesting a shift in the metabolic mechanisms as biomass builds up. The previously mentioned new flocculent strains were applied in a high cell density concentric tube airlift bioreactor [118]. In continuous operation, an ethanol productivity of 11 $g \cdot L^{-1} \cdot h^{-1}$ was obtained (with a feed lactose concentration of 50 g/L and a dilution rate of 0.55 h^{-1}), being seven-fold larger than the one in conventional continuous systems. Despite the flocculence instability of the recombinant strain, a high biomass concentration was achieved inside the bioreactor as its design allowed for a selection of the most flocculating cells from a mixed culture, contributing thus with a selective pressure for the maintenance of the flocculating cells inside the bioreactor. The most direct application of this work is the high-productivity fermentation of the lactose present in cheese whey to produce ethanol, not only contributing to the bioremediation of this by-product of the dairy industry produced in large amounts, but also allowing for the production of a useful fuel.

One of the shortcomings of flocculation bioreactors, already mentioned, is the presence of mass transfer limitations inside the flocs. It is possible to calculate a floc critical diameter, defined as the value of diameter at which solid phase diffusion limitations become more important than liquid phase diffusion limitations [147]. A floc diameter greater than its critical value can have consequences such as the presence of useless biomass inside the reactor, undesirable metabolite production by inactive biomass, changes in chemical and biochemical characteristics of medium and microorganisms, suggesting a continuous disintegration of the flocs into a smaller size. In the same line, Webster [154] developed criteria allowing for the assessment of the importance of substrate diffusional limitations within cell flocs, depending on the rate law used to describe substrate consumption. Thus, a reduction in floc size could be expected to bring a reduction in mass transfer limitations, leading to an increase of productivity. Vicente *et al*. [155] introduced static mixers in the draught tube of the internal loop airlift bioreactor used by Sousa *et al*. [126,127], achieving an effective reduction of the floc size (3 mm to 1 mm in diameter). Steady state data at different dilution rates were measured for both systems (the original and the modified bioreactor) and the results were compared in terms of specific consumption/production rates and ethanol productivity. A 40% increase was obtained in the maximum dilution rate at which a glucose conversion higher than 98% could be achieved. The respiratory quotient had a constant value (around 23) at all dilution rates, meaning that the meta-

Table 1. Scientific work using flocculation bioreactors

Organism	Reactor type	Volume [L]	Main substrate	Main product	Productivity [$g \cdot L^{-1} \cdot h^{-1}$]	Ref.
S. cerevisiae	bubble column	1-50	wort	beer	-	[157]
S. cerevisiae	bubble column	-	molasses	ethanol	25-30	[164]
K. marxianus	external loop airlift	1.2	lactose	ethanol	24.4	[117]
S. cerevisiae	external loop airlift	2	glucose / sucrose	ethanol	68	[165]
S. cerevisiae	CSTR	3	glucose	ethanol	5	[166]
S. cerevisiae	series of 2 CSTR	0.5 + 1.5	molasses	ethanol	9.3	[167]
S. cerevisiae	internal loop airlift	5.4	glucose	ethanol	-	[127]
S. cerevisiae	external + internal loop airlift	1.2 + 5.4	glucose	ethanol	12.9	[126]
S. cerevisiae	fluidised bed	10	molasses	ethanol	15- 20	[168]
S. cerevisiae	external loop airlift	2	sucrose	ethanol	-	[169]
S. diasctaticus	external loop airlift	2	Jerusalem artichoke extract	ethanol + inulin	-	[170]
Z. mobilis + *Saccharomyces sp.*	agitated conical flasks	1	-	ethanol	1.5	[171]
Rhodiola sachalinensis	internal loop airlift	10-100	-	salidroside	-	[172]
S. cerevisiae	internal loop airlift	6	green beer	maturated beer	-	[173]
Schizo-saccharomyces pombe	external loop airlift	1.2	grape musts	deacidified grape musts	-	[60]
K. marxianus	internal loop airlift	1000	deproteinised cheese whey	ethanol	-	Unpublished data
Recombinant *S. cerevisiae*	internal loop airlift	6	lactose	ethanol	-	[118]
Recombinant *S. cerevisiae*	internal loop airlift	6	deproteinised cheese whey	ethanol	-	[174]

bolic state of the cells in flocs remained constant, having a strong fermentative metabolism.

The floc size reduction contributed to the higher observed reaction rates, not only by means of an increased dilution rate, but also because of reduced diffusional limitations, leading to a 30% increase of ethanol productivity when compared with the original system.

Applications of Flocculation Bioreactors

One of the first commercial applications of the sedimentation characteristics of flocculating microorganisms was made by the brewing industry, back in 1971, in order to facilitate the separation of the yeast cells from beer at the end of the process [156]. Still, it is mostly in the brewing industry that flocculation bioreactors are widely used. However, in this case, flocculation is essentially a separation technique and not a way to immobilise cells in continuous high cell density systems. Despite this, the works with flocculating bioreactors are being performed for some decades, as Smith and Greenshields [157] have successfully grown flocculent strains of brewing yeast in bubble column fermenters. Most brewing companies and brewing research groups have several research works using high cell density bioreactors, mostly with airlift configuration, in order to investigate their potential use in continuous beer fermentation, with the advantages pointed out throughout the preceding text [158-163]. Nevertheless, none of these works actually deals with flocculating cultures, though some mention them as a possible alternative to the existing processes, in particular for beer maturation [158].

In Table 1, a summary of works with flocculation bioreactors is presented, proving an emerging interest for this type of systems. As can be seen, the majority of the work presented deals with flocculating microorganisms for continuous ethanol production, which is not surprising since, for the moment, continuous high cell density systems are adequate for high volume low added value products. Recently, protein secretion by flocculating yeast cells has been reported as another possible application for high cell density bioreactors [115].

As also indicated, most of the studies on flocculation bioreactors have been done in bench-scale apparatuses due to costs and complexity involved in larger scale research. Being so, further information on hydrodynamics and mass transfer needed for reactor scale-up is still missing and it is not surprising that, so far, industry still hesitates to select a flocculation-based process for commercial purpose, in spite of the operational advantages of these systems.

CONCLUSION

Known for several years, yeast flocculation is a phenomenon that has not yet been fully clarified. To date,

three mechanisms have been invoked to explain the yeast flocculation: the colloidal theory, the Ca^{2+} bridge theory and the lectin-model theory. The lectin-model theory is the widely accepted model but, as suggested by Speers *et al*. [63], it may happen that more than one of these models is operative, as experimental data accumulate indicating that different mechanisms for flocculation exist in different yeast strains and environmental conditions.

At industrial scale, only beer producers have taken advantage of yeast flocculation ability by using this property to clarify the fermented must. This is a rather limited application of flocculating yeast cells as, at laboratory and pilot plant scale, the advantages of continuous bioreactors for the production of ethanol (a primary metabolite) have been clearly demonstrated. More recently, the advantages of these bioreactors for the production of an enzyme have also been demonstrated, indicating the potential use of flocculent yeast cells in the emerging proteins, vitamins and co-factors production through recombinant DNA technology. Flocculating properties of yeast cells can be used either to increase overall productivity in continuous bioreactors by allowing for high biomass retention or to facilitate downstream processing in continuous, fed-batch or batch reactors.

In conclusion, it may be said that there is the need for more research on flocculent yeast strains and high cell density systems, in general, and on flocculation bioreactors, in particular, in order to gather the necessary information to make them an interesting alternative to the processes used nowadays, which are in most cases very well established and studied. The potential surely exists in this new technology, but it has to be demonstrated before the industry risks investing largely in it.

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