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PLASMID STABILITY IN RECOMBINANT *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

Because of many advantages, the yeast *Saccharomyces cerevisiae* is increasingly being employed for expression of recombinant proteins. Usually, hybrid plasmids (shuttle vectors) are employed as carriers to introduce the foreign DNA into the yeast host. Unfortunately, the transformed host often suffers from some kind of instability, tending to lose or alter the foreign plasmid. Construction of stable plasmids, and maintenance of stable expression during extended culture, are some of the major challenges facing commercial production of recombinant proteins. This review examines the factors that affect plasmid stability at the gene, cell, and engineering levels. Strategies for overcoming plasmid loss, and the models for predicting plasmid instability, are discussed. The focus is on *S. cerevisiae*, but where relevant, examples from the better studied *Escherichia coli* system are discussed. Compared to free suspension culture, immobilization of cells is particularly effective in improving plasmid retention; hence, immobilized systems are examined in some detail. Immobilized cell systems combine high cell concentrations with enhanced productivity of the recombinant product, thereby offering a potentially attractive production method, particularly when nonselective media are used. Understanding of the stabilizing mechanisms is a prerequisite to any substantial commercial exploitation and improvement of immobilized cell systems.

Key words: Recombinant yeast, *Saccharomyces cerevisiae*, plasmid stability, immobilized cells, recombinant *Escherichia coli*, recombinant culture.

INTRODUCTION

From inception only two decades ago, recombinant DNA technology has made spectacular advances. Many recombinant products are already on the market, and many more are about to reach commercialization. Genetic 'engineering' offers immense possibilities for manufacture of exotic and valuable substances that were virtually unobtainable before. Recombinant products are usually expressed in microorganisms—mostly bacteria and yeasts—to achieve high yields by taking

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advantage of the rapid microbial growth rates. Earlier work on recombinant DNA technology focused mostly on prokaryotes such as *Escherichia coli*, but it was soon obvious that bacteria were far from the ideal host. Attempts to find a more suitable host led immediately to the well known yeast *Saccharomyces cerevisiae*, bakers' or brewers' yeast. Unlike *E. coli*, *S. cerevisiae* lacks detectable endotoxins and it is 'generally recognized as safe' because of a long history of use in food and pharmaceuticals. Genetic manipulation of *S. cerevisiae* has been made easier with greatly improved understanding of the yeast's biology and genetics. Recombinant proteins can be expressed in the yeast, correctly folded, and modified at the posttranslational level to yield a biologically active product. Furthermore, the process technology for yeast culture is well established [1], and it can be easily adapted to producing recombinant proteins. Finally, expression in yeast may allow natural extracellular release of the protein because of a secretion system that is similar to that of higher eucaryotes. Biologically active, secreted products are substantially easier to recover than the denatured, inclusion body types usually produced by recombinant bacteria [2, 3]. Other important process advantages of recombinant yeast relative to bacteria have been noted by Garrido et al. [4]. Because of all these advantages, *S. cerevisiae* is now an established and often preferred host for expression of recombinant proteins. Many recombinant proteins, including commercial products, are produced in *S. cerevisiae* [3–8].

In commercial production with recombinant microorganisms, the most important problem is plasmid instability. Instability is the tendency of the transformed cells to lose their engineered properties because of changes to, or loss of, plasmids. This problem is especially significant with recombinant yeasts because almost all yeast plasmid vectors are hybrid plasmids, or 'shuttle vectors,' that are relatively unstable. Plasmid instability may be of two types: structural instability and segregational instability. Structural instability is usually caused by deletion, insertion, recombination, or other events, at the level of the DNA; whereas segregational instability is caused by uneven partitioning of plasmids during cell division [9]. Genetic instabilities in combination with environmental factors lead to plasmid loss. During culture, cells lacking the plasmid appear, coexisting and competing with the plasmid-containing population. Unfortunately, the plasmid-free cells usually have a growth advantage

over plasmid-containing cells in nonselective media because the extra plasmid places additional metabolic load on the host [10]. Repeated batch cultures or continuous cultures select for the most competitive cells; hence, in time, the plasmid-free cells overwhelm the original plasmid-bearing population. Consequently, plasmid instability results in massive loss of productivity of the desired product, thus, being a major hurdle to large scale industrial use of the genetically modified microorganisms.

Development and optimization of recombinant yeast fermentations, and design of better vectors, require knowledge of the genetic and environmental factors that determine stability. Mechanisms of instability need elucidation, as do the dynamics of interactions between the host cell and the plasmid. This monograph reviews the state of knowledge on plasmid stability in the recombinant yeast system, and identifies areas where further work is especially needed. Ultimately, such knowledge would determine the feasibility of large scale culture of recombinants.

PLASMID VECTORS

Almost all yeast plasmid vectors are shuttle vectors. They are, in fact, both yeast and *E. coli* vectors, that can replicate in either organism. Such vectors usually consist of all or part of an *E. coli* vector, for example pBR322, and a yeast replication system. Because of this arrangement, *E. coli* can be employed as the 'preparative' organism for the many procedures required in genetic manipulations. Yeast autonomous plasmid vectors are based on one of two replication systems: either the sequences derived from the endogenous yeast 2 μ m plasmid are used; or chromosomal DNA fragments that can replicate autonomously are employed [11, 12]. The latter type are usually the autonomously replicating sequences (ARSs). Such sequences presumably act as origins of replication in yeast chromosomes. Several different types of yeast vectors have been reported [13]; the principal ones are: (i) yeast integrating plasmids (YIp); (ii) yeast episomal plasmids (YEp); (iii) yeast replicating plasmids (YRp); (iv) yeast centromeric plasmids (YCp); and (v) yeast linear plasmids (YLP). These groups are briefly described below.

Yeast Integrating Plasmids (YIp)

In principle, a yeast cell may be transformed with any piece of DNA, or even with a

mixture of DNA fragments. A subsequent recombinant event integrates the foreign DNA into the yeast genome. Early research employed this procedure. However, random recombination is rare, and the introduced DNA is rapidly degraded in the cell. This method was improved by Hinnen et al. [14] into a new, revolutionary transformation system: a bacterial plasmid pYeLeu10 linked to a yeast *leu2* gene was transformed into a *leu2* auxotrophic strain; the plasmid integrated at the homologous *leu2* region in the yeast chromosome. Such yeast integrating plasmids (YIp) show a low transformation efficiency, but result in stable transformants. However, only one or a few gene copies per cell may be integrated.

Yeast Episomal Plasmids (YEp)

Because the YIp plasmid lacks sequences for autonomous replication, it cannot replicate on its own. To resolve this problem, Beggs [15] inserted the replication origin of the endogenous yeast 2 μ m plasmid into the transformation vector. The resulting circular vector (YEp plasmid) no longer integrated into the chromosome, but replicated on its own. YEp plasmid shows a high transformation frequency, and a high copy number, but it is less stable than the YIp transformants.

Yeast Replicating Plasmids (YRp)

Replacement of the replication origin of the 2 μ m yeast plasmid by an autonomous replicating sequence results in a yeast replicating plasmid (YRp). YRp vectors have high transformation frequency, but are even less stable than the YEp vectors; however, the copy number is usually high.

Yeast Centromeric Plasmids (YCp)

Clarke and Carbon [16] improved the stability of the YRp plasmids by adding yeast chromosomal centromere sequences, hence, giving rise to YCp plasmids. YCp plasmids show high transformation efficiency, but the copy number is reduced to one per cell.

Yeast Linear Plasmids (YLp)

YRp plasmids can be linearized by the addition of telomers originating, for example, from the yeast. The resulting plasmid is a yeast linear plasmid (YLp). The copy number, the transformation frequency, and the stability are similar to those of the YRp.

PLASMID STABILITY

After recombinant plasmids are introduced into the host yeast, the interactions between plasmid and the host are substantial. These interactions determine the stability of plasmids, and the expression extent of the cloned genes. Factors that affect these interactions may influence plasmid stability. Compositions and properties of plasmid vectors themselves affect stability, and so do physiological and genetic characteristics of the host, as well as the environmental factors. Those genetic and environmental influences are summarized in Table 1, and discussed in detail in the following sections.

Genetic Factors

Plasmid DNA sequence effects. Plasmid stability may be enhanced by including stabilizing genes in plasmid vectors. The 2 μ m DNA-based vector is the best studied in *S. cerevisiae*. The replication loci *rep1* and *rep2* of the 2 μ m have been associated with stability during replication of the native plasmid [13]. Futcher and Cox [17] have

Table 1. Factors affecting plasmid stability in recombinant yeast.

Genetic Factors	Plasmid makeup Plasmid copy number Expression level Selective markers Properties of host cells
Environmental Factors	Medium formulation Dissolved oxygen tension Temperature Dilution rate Bioreactor operation modes

shown that plasmids containing both *rep1* and *rep2* are considerably more stable than those lacking one or both loci. Inclusion of chromosomal centromere regions in vectors has also been used to construct stable plasmids. One such plasmid YRp7, containing autonomous replication sequences, is fairly unstable in *S. cerevisiae*; however, once a centromere region, *cen*, carrying partition function is inserted into YRp7, the plasmid becomes stable [16]. Less than 5% of such vectors are lost after 20–40 generations under nonselective conditions. Plasmids can be stabilized by joining with a particular DNA segment that provides the good partition function for *E. coli* [18]. When the partition locus of plasmid pSC101 was added to both pBR322 and pACYC184, the stabilities of the resulting plasmids increased by 3- to 10-fold [18]. For the yeast system, the *stb(rep3)* sequence, in conjunction with the *rep1* and *rep2* gene products encoded by the endogenous 2 μ m plasmid, ensures a high degree of partition stability [19]. In another study, the insertion of a λ *cos*-containing DNA sequence into one of the pSa plasmids increased the fitness and growth rate of the host cells carrying this λ *cos* pSa construct [20]. Inclusion of certain genes can enhance plasmid maintenance by coupling host cell division to plasmid proliferation [21]. Although those experiments were done in *E. coli*, the methods developed may be applicable to *S. cerevisiae*. Presence of certain other genes in plasmids can adversely affect plasmid stability. Kuriyama et al. [22] reported that when *S. cerevisiae* AH22R⁻-2075 harboring pGLD p31-RcT which contains rHBsAg P31 mutein coding gene and β -isopropylmalate dehydrogenase gene (*leu2*) was cultured on large scale in a leucine-devoid medium, plasmid-free cells appeared at high frequency. The plasmid instability was thought to have been caused by the insertion of the *leu2* gene into endogenous yeast 2 μ m DNA as a result of homologous recombination between 2 μ m DNA and the expression plasmid [22]. As these examples prove, the genetic make-up of a plasmid can greatly affect its stability.

Plasmid copy number. Stability of vectors in *S. cerevisiae* depends on the plasmid copy number. Because the desired gene is usually inserted into the plasmid vector, the copy number of the plasmid determines the gene dosage in the cell. In general, plasmids showing high copy number exhibit greater stability than those with low copy

number [17]. During cell division, efficient partitioning of plasmids between mother and daughter cells will prevent emergence of plasmid-free cells. On the other hand, inefficient partitioning will result in plasmid-free cells. Based on this concept, Walmsley et al. [23] reasoned that a plasmid with a high copy number should generate plasmid-free cells much less frequently than a plasmid with lower copy number. However, opposing results have been observed. For example, the 2 μ m plasmid is more stable than either YEp plasmid or YRp plasmid even though the latter is usually present in higher copy number [24]. Spalding and Tuite [24] further reported that the stability of the YRp plasmid (YRp7M) in a haploid strain was significantly less than that of the YEp plasmid (pMA3a), although the former had an approximately sevenfold higher copy number. This apparent inconsistency occurs because YRp plasmids have no effective plasmid-encoded partition system, and show a strong segregation bias toward the mother cell during mitotic division [24]. Thus, a high copy number does not necessarily guarantee stability unless the plasmid has an effective partitioning system. The latter depends on the efficiency of the origin of replication. Effective ori regions consist of two fundamental domains: a replication-inducing sequence, and a replication-enhancer sequence. The enhancer sequence is not essential, but ensures optimal stability of the ori-containing vector [25]. The efficiency of 2 μ m origin is higher than that of *ars1* origin [24], as was confirmed also by Da Silva and Bailey [26]. The cloned gene product's synthesis was much lower with the *ars1* plasmid than with the 2 μ m based plasmid because of the smaller fraction of plasmid-bearing cells (7.5%) when the plasmid's origin of replication was the *ars1* element [26]. Increased stability with increased copy number has been reported for 2 μ m based plasmids [27]. Clearly, the effect of copy number on plasmid stability relates to the origins of replication. Stability depends on efficiency of the origin of replication, and on the copy number.

Expression level or transcription efficiency effect. Parker and DiBiasio [28] used an auxotrophic mutant of *S. cerevisiae* containing a recombinant 2 μ m based plasmid to examine the effect of the plasmid expression level in continuous culture. The plasmid introduced the ability to synthesize acid phosphatase that had been

deleted from the host. The *pho5* promoter present in the plasmid controlled the transcription of acid phosphatase (expression level) by responding to concentrations of inorganic phosphate. Expression level was measured via the activity of acid phosphatase. As the level of plasmid expression was raised, the plasmid stability declined markedly [28]. This was probably because an increased transcription repressed the replication of plasmid, increased segregational instability, and overburdened the cell's capability to repair DNA [28]. Furthermore, the known toxicity toward the host of large amounts of some foreign proteins [29] may also contribute to plasmid loss.

Transcription efficiency can be controlled by the promoter strength and regulatory mechanism in a given host cell. Plasmids with inducible promoters may be used to overcome the adverse effects of cloned gene expression. Such inducible promoters can be switched to control the time and the level of gene expression [26]. Da Silva and Bailey [26] used the yeast's galactose regulatory circuit to study the influence of promoter strength on plasmid stability in batch and continuous culture. The *gal1*, *gal10*, and *gal10-cycl* yeast promoters, included in plasmids pRY121, pRY123, and pLGSD5, respectively, were employed. Cloned *lacZ* gene expression was regulated by the promoters through addition of galactose. Higher β -galactosidase production, lower growth rate, and reduced plasmid stability were observed for the strain bearing the plasmid with the strongest *gal1* promoter [26]. Although a high expression level can be detrimental to plasmid stability and cell growth, in this particular case the reduced plasmid stability and growth rate were more than offset by increased enzyme specific activity, and productivity [26]. In large scale batch or continuous fermentations, plasmid instability is fatal; hence, high expression levels that result in instability are not wanted. Modulation of gene expression at an optimal level demands easily controlled promoter systems. Relationships among expression level, promoter system, and plasmid stability need to be further examined.

Selective markers. Unless a plasmid confers an obvious phenotype on the harboring cells, a selection marker is necessary for identifying the transformed clones. The yeast 2 μ m plasmid confers no overt phenotype. Presence of a selectable marker

allows for stable maintenance of the plasmid if a selection pressure is imposed. Genes that confer resistance to antibiotics are commonly used markers. Such genes are inserted into plasmids, and the resulting vectors are used for transformation. Antibiotics-containing selective media are now used to eliminate plasmid-free cells while stably retaining the transformed cells. Many commonly used antibiotics are ineffective against yeasts; one effective compound is the aminoglycoside geneticin G418. This antibiotic is inactivated by the enzyme aminoglycoside phosphotransferase-3'(I) that is coded by the bacterial transposon Tn601. Yeast transformed with a plasmid carrying Tn601 becomes resistant to G418 at concentrations greater than $150 \mu\text{g}\cdot\text{mL}^{-1}$ [30]. Other selection markers have been used to confer resistance to antibiotics such as methotrexate [3, 31] and chloramphenicol [32]. However, use of antibiotics is not free of pitfalls. Antibiotics are expensive, and their presence complicates product recovery. Sometimes, for example in wastewater treatment with recombinants [33], use of antibiotics may be totally impractical. Another selectable marker is the *cup1* gene that imparts resistance to high levels of copper. This gene has been cloned and characterized [34]; it offers a potentially useful alternative to antibiotics.

The genes coding for enzymes in the amino acid biosynthetic pathway are also commonly employed as selection markers. Two such markers are *leu2* and *trp1*. Host mutants for leucine and tryptophan auxotrophy are easily selected. The plasmid carrying *leu2* or *trp1* is cloned into the auxotrophic mutant. Plasmid retention is then necessary for survival of the auxotroph if the medium is devoid of essential leucine or tryptophan. This system requires the use of a defined selective growth medium, a limitation that is overcome in autoselective systems. The latter combine a host mutant that lacks a gene that is essential for survival with a plasmid vector carrying the essential gene. Thus, without the plasmid, the mutant cell dies regardless of the culture medium and conditions. *S. cerevisiae* autoselective strains with mutation in the *ura3*, *fur1*, and *urid* genes have been obtained through sequential isolation [35]. Those mutations effectively block both the pyrimidine biosynthetic and salvage pathways, and, in combination, are lethal to the host. Therefore, a plasmid carrying a *ura3* gene is essential for survival, and nonselective media can be employed without the risk of

plasmid loss [35]. A more advanced autoselection system was described by Lee and Hassan [36]. In this case, the selection pressure was imposed by utilizing the yeast killer toxin-immunity complementary DNA inserted into a plasmid. Thus, presence of the plasmid vector conferred not only toxin immunity, but the selective agent (killer toxin) was secreted into the culture fluid. The plasmid-carrying cells survived because of immunity, but the plasmid-free cells perished. Lee and Hassan [36] transformed the plasmid (pYT760-ADH1) containing the yeast killer toxin-immunity cDNA into a leucine-histidine mutant (AH22), and showed that the plasmid was extremely stable. This system has the potential to operate in any strain that is initially sensitive to killer toxin. Because autoselection systems for plasmid maintenance function in any growth medium, such systems are ideal for large scale industrial culture of recombinants.

Host cell characteristics. Interactions between the host cell and the plasmids certainly play an important role in maintenance of the plasmid; hence, the properties of the host must also be considered in any discussion of plasmid stability. A certain plasmid transformed into different mutants of a host may display different stability characteristics in different mutants [37, 38]. The host mutants may affect plasmid partitioning, replication, or amplification, all of which relate to stability. Some evidence suggests that the plasmids may utilize some form of anchorage to a host cellular structure, hence leading to possible asymmetric inheritance upon cell division [39].

The growth cycle of a host greatly affects the processes of replication and transcription of plasmid, and that relationship is bound to affect plasmid stability. Unfortunately, there is little data on the effects of physiological properties on plasmid stability, and some results are inconsistent. Mead et al. [40] observed that the rate of plasmid loss was reduced by allowing haploid populations to enter stationary phase periodically. In contrast, for the 2 μ m based yeast hybrid plasmids, Kleinman et al. [41] reported reduced stability in the stationary phase. Growth rate in the stationary phase is usually minimal, and effects of specific growth rate on plasmid stability have been examined as discussed in later sections of this review.

Manipulating the ploidy of the host cell also has an effect on plasmid stability. For the highly unstable, ARS-based plasmid YRp7M, a significant increase in

segregational stability was observed with increasing ploidy, while the relatively stable, 2 μ m based plasmid pMA3a showed only a slight increase in stability in strains of higher ploidy [24]. Greater plasmid stability in diploids was reported also by Mead et al. [40]. Industrial yeast strains for brewing and baking are usually polyploid.

Attempts to improve plasmid stability have focused mainly on manipulating the plasmid; little attention has been paid to the genetic constitution or the physiological state of the host. Manipulating host cells is certainly an option for improving plasmid stability. Much work remains to be done in this area.

Environmental Factors

Cell's response to the environment originates ultimately at the genetic level; hence, the culture conditions profoundly affect plasmid stability and expression (Table 1). Control and manipulation of the environment are particularly relevant to large scale culture where the feasibility of production depends on the cost of providing the requisite environment. Moreover, once a suitably engineered host is selected for production, environmental manipulation is the sole remaining option for maintaining stability. A discussion of the main environmental influences on plasmid stability follows.

Medium formulation. Metabolic activities of microorganisms are strongly influenced by the composition of culture media. For recombinant cells, medium composition can affect the stability of plasmid through different metabolic pathways and regulatory systems of the host. In some cases, media that provide selection pressures are essential to maintaining plasmid stability (see Genetic Factors). Generally, minimal media favor stability relative to richer media [37]. Wang and Da Silva [42] reported on effects of three media on plasmid stability in recombinant *S. cerevisiae* strain SEY2102/pSEY210, that produced invertase. A minimal medium (SD), a semidefined medium (SDC), and a rich complex medium (YPD) were investigated. Invertase productivity did not improve as the medium was enriched from SDC to YPD. In the complex YPD medium, the plasmid stability dropped from 54% to 34% during a single batch fermentation [42]. During long-term sequential batch

culture in YPD, the invertase activity decreased by 90%, and the plasmid-containing fraction of the population declined from 56% to 8.8% over 44 generations [42].

Dissolved oxygen tension. A supply of oxygen is essential for most commercially relevant microorganisms. Availability of oxygen may affect growth rate, in addition to having other complex effects on metabolic pathways. Effects of dissolved oxygen on microbial metabolism have been extensively described [1, 43–45]; however, there is little information on how oxygen affects expression and stability of plasmids. Tolentino and San [46] showed that plasmid stability in recombinant *E. coli* was unaffected by dissolved oxygen levels. The plasmid was stable during a step change in which the oxygen supply was replaced with nitrogen. In contrast, the concentration of dissolved oxygen affected plasmid stability in a recombinant yeast [47]. Using a glucose-limited chemostat culture, Lee and Hassan [47] examined the effects of oxygen tension and dilution rate on stability and expression of killer toxin plasmid (pADH-10A) in wine yeast (MONtrachet 522). The recombinant yeast was grown in nitrogen-, air-, and pure oxygen-sparged environments. The highest plasmid stability was observed in the air-sparged culture, suggesting the possibility of an optimum dissolved oxygen concentration for greatest stability of the plasmid. Similar results were reported by Caunt et al. [48] in studies of oxygen limitation on plasmid stability in recombinant yeast grown in a nonselective medium. The yeast strain was YN124/pLG669-z, which produced β -galactosidase. Once the dissolved oxygen level was lowered to below 10% of air saturation, the fraction of plasmid-containing cells declined sharply [48].

Although *S. cerevisiae* can grow fermentatively, replication and transcription of a multicopy plasmid require large amounts of energy. Failure to provide sufficient oxygen will reduce the energy supply, possibly affecting replication and partitioning of the plasmid. Further work is necessary on the role of oxygen on plasmid stability.

Temperature. Temperature affects microbial growth rate, biosynthetic pathways and directions, and regulatory systems. Such effects are particularly significant in recombinant fermentations. In one case, the optimal temperature for production of recombinant proteins—interferon and insulin—in *E. coli* was reduced to only 30°C,

whereas the optimal growth temperature for the wild host is 37°C [49]. Temperature can also affect plasmid stability, but little is known about this aspect, especially in recombinant yeast. Relatively more information is available for recombinant *Bacillus subtilis* and *E. coli* systems. Stability of pTG201 in *E. coli* depends strongly on the culture temperature [50]. At 31°C, the plasmid was stable: after 83 generations more than 82% of cells contained plasmid [50]. However, at 37°C, only about 40% of cells retained plasmid after about 87 generations. At a yet higher temperature (42°C), only about 35% of the population had plasmid after roughly 67 generations. In these studies, the initial percentage of cells carrying pTG201 at 31, 37, and 42°C was, respectively, 97, 85, and 94%. Similar results were noted for the same plasmid in another host strain *E. coli* W3101. Plasmid stability in a recombinant *Bacillus* was shown to decline when temperature rose above 30°C [51]. In continuous culture of *Bacillus subtilis* (pHV1431) without selection pressure, the plasmid was segregationally less stable at 30°C than at 37°C, but no structural instability was observed at either temperature [52].

Dilution rate. Effect of dilution rate on plasmid stability in continuous culture has been extensively studied. Because the dilution rate corresponds to the specific growth rate in continuous culture, the chemostat culture provides an easy method for elucidation of the relationships between growth rate and plasmid stability. In several studies plasmid stability increased with increasing dilution rate when recombinant *S. cerevisiae* was continuously cultured in selective media [26, 28, 36, 47, 48, 53]. When an auxotrophic mutant of *S. cerevisiae* containing a recombinant 2 μ m plasmid was grown in selective media, Parker and DiBiasio [28] observed that plasmid stability substantially increased at higher growth rate. Stability of a plasmid that contained killer toxin cDNA increased as a function of dilution rate in chemostat cultures of *S. cerevisiae*; the plasmid was fully stable once the dilution rate exceeded a certain value [36]. These results on the dilution rate effect are remarkably consistent considering the different yeast strains that were tested. The effect of dilution rate seems to relate to selection pressure in the medium. In a nonselective medium, increasing dilution rate

was accompanied by increasing segregational instability; however, in a selective medium, at high dilution rate, the fraction of plasmid-containing cells remained relatively constant for about 100 generations [54]. Impoosup et al. [55] examined the stability of 2 μ m based yeast plasmid (pLG669-z) during continuous culture with cyclic growth rate variations in a nonselective medium, and showed that plasmid stability fell at high dilution rate. The dilution rate was cycled between a very low growth rate (0.075 h^{-1}) and a higher growth rate (0.25 h^{-1}). During growth at the high dilution rate, the fraction of plasmid-containing cells and the expression fell [55]. After the dilution rate was changed to the low value, the plasmid-bearing fraction and expression level rose almost to the initial values [55]. Higher stability of a yeast plasmid at lower dilution rate in nonselective media was observed also by Caunt et al. [56]. Attempts have been made to explain these effects theoretically at the molecular and cellular levels, as well as through macroscopic models and simulations [28, 53, 57–60], but actual mechanisms remain unclear.

The degree of plasmid instability is related to the probability of plasmid loss (p) and/or mutation per division of host cells [61]. A second factor that affects stability is the growth ratio α . The latter is defined as the ratio of specific growth rate of plasmid-free cells (μ^-) to that of plasmid-containing cells (μ^+). The probability of plasmid loss depends largely on genetic factors; whereas the growth ratio is affected by both genetic and environmental factors. The larger the probability of plasmid loss and the growth ratio, the less stable the plasmid. The effect of dilution rate on plasmid stability may be explained by those two parameters. In chemostat culture, the dilution rate equals the specific growth rate which can affect the growth cycle. Dilution rate dependence of specific growth rate was invoked by Parker and DiBiasio [28] in attempts to explain effects of dilution rate on plasmid stability. Replication and transcription of plasmid were assumed to have the potential to damage the plasmid DNA [28]. In this scenario, plasmid stability would depend on the cell's DNA repair capabilities. Because many of the enzymes required for DNA repair are synthesized periodically, the repair capability is likely to vary with the stage of growth cycle. Figure 1 illustrates the cell cycle of budding yeast as presented by Hjortso and Bailey [57]. Enzymes such as DNA ligase, DNA polymerase I, and nucleases have high

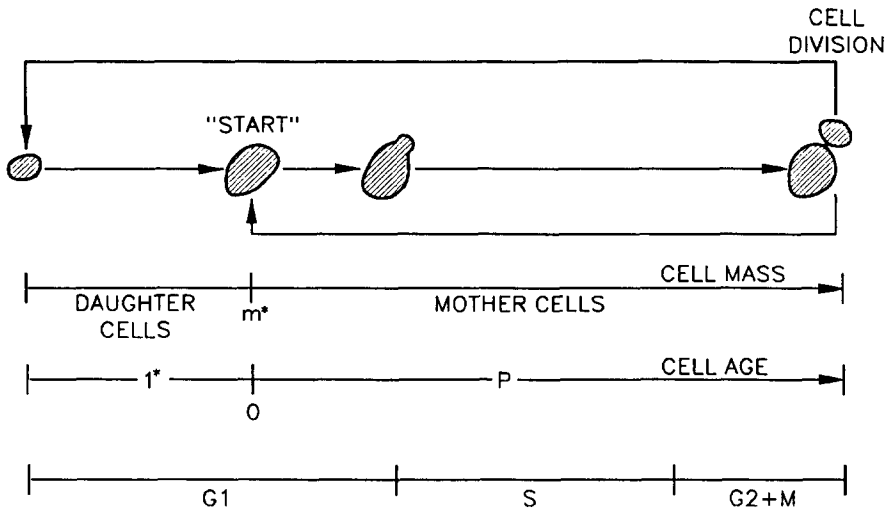


Figure 1. Cell cycle of budding yeast. Based on Hjortso and Bailey [57].

levels in the S and G_2 phases. When growth rate is enhanced through manipulation of the dilution rate, the S and G_2 phases comprise a significantly larger portion of the cell cycle; consequently, the cell's DNA repair capability is high. However, the need for repair remains great at low growth rates when the rates of replication or transcription are large. During slow growth, the long G_1 phase relative to G_2 and M phases could compromise the cell's ability to complete the necessary repairs, hence increasing the likelihood of loss and mutation.

Although the foregoing reasoning does explain the effect of dilution rate on plasmid stability in selective media, it does not explain that effect in nonselective media. An alternative possible explanation follows. For continuous culture in selective media, increase in dilution rate raises the specific growth rate of plasmid-harboring cells according to the well known equation

$$\text{dilution rate} = \mu^+ = \mu_m^+ \frac{S}{K_s + S} ; \quad (1)$$

however, plasmid-free cells do not grow, or grow very slowly, because of the selection pressure. Therefore, the growth ratio ($\alpha = \mu^-/\mu^+$) decreases as the dilution

rate is increased, so the plasmid is more stable. In nonselective media, because the growth rate of recombinants is adversely affected by plasmid load and expression of foreign protein [10], plasmid-free cells usually have a growth advantage. Commonly, the growth ratio α exceeds unity, ranging over 1 to 2 [61]. Increasing dilution rates in nonselective media can further raise the growth ratio, because the plasmid-bearing cells are less able to respond to the increase. Therefore, loss of plasmid stability with increasing dilution rate in nonselective media is not surprising. In fact, in continuous culture of recombinants, a steady state cannot be achieved in the absence of selection pressure if the growth ratio exceeds unity.

Bioreactor operational schemes. Fermentation processes are usually operated as batch, fed-batch, or continuous culture. The mode of operation can influence the plasmid stability and the productivity of expressed products. In batch culture, plasmids are relatively stable because the culture periods are generally short, hence, the number of generations since inoculation is small. Because of long growth periods, continuous culture of recombinant cells suffers from plasmid instability particularly in nonselective media [53, 55, 62]. A two-stage continuous culture system based on inducible operators has been devised in attempts to overcome the instability problem [50, 63, 64]. In this scheme, a repressor protein binds to the operator of the plasmid to block transcription until needed. When necessary, an inducer is added, to bind to the repressor, and prevent its interaction with the operator, thus allowing transcription to begin. In the first stage of the two-stage system, the cells are grown in the repressed state (no foreign protein is synthesized) or under selective pressure in order to prevent plasmid loss, and obtain a high fraction of plasmid-containing cells. The cells are then continuously transferred to the second stage, where the inducer is added to enable expression. Another possibility for maintaining plasmid-harboring cells in continuous culture is the use of selective recycle [65]. In such a process, cells leaving the reactor are concentrated and returned to the reactor, resulting in increased cell concentration and product synthesis. Feasibility of selective recycle depends on the ability to easily separate the plasmid-containing cells from the mixed population; therefore, the plasmid phenotype must include a property than can be the basis of separation. Such a property may be cell size, density, or flocculation behavior.

Another promising stabilizing option is cycling of operational variables. Using a theoretical analysis, Stephens and Lyberatos [60] showed that cycling of substrate concentration could lead to coexistence of plasmid-containing and plasmid-free cells in continuous culture. In an experimental study with *E. coli* K21 strain grown in nonselective continuous culture, Weber and San [66] observed that steady dilution rates over time led to declining fraction of plasmid-bearing cells. Eventually, the plasmid-free cells displaced the recombinants. However, when dilution rate oscillated, the reactor maintained a mixed population of plasmid-free and plasmid-containing cells for longer periods. For recombinant yeast, Caunt et al. [48, 56] showed that cycling of dissolved oxygen with a frequency of a few minutes could enhance plasmid stability. Whereas Impoolsup et al. [55] observed that cyclic variations in dilution rate had a stabilizing effect on the 2 μ m based yeast plasmid. Stabilizing effects of growth rate (dilution rate) cycling may be simulated in fed-batch fermentations by controlled intermittent feeding [55]. Plasmid stability of recombinant *S. cerevisiae* YN124/pLG669-z in fed-batch culture was examined by Hardjito et al. [54]. The fraction of plasmid-containing cells could be maintained constant over the length of one fed-batch culture, suggesting that fed-batch processes were better suited to producing recombinant proteins. Although the effects of cyclic environmental changes on plasmid stability are clear, the reasons for those effects are not fully understood. Newer bioreaction strategies are being developed continually to improve productivity. One such example is the airlift bioreactor [45] that is particularly capable of microenvironmental cycling within a mechanically robust, simple, and scalable device. Little information exists on the use of airlift devices in large scale culture of recombinants, even though airlift bioreactors are thoroughly proven in the fermentation industry [45].

IMMOBILIZED CELL SYSTEMS

Immobilized enzymes and cells have been widely employed to enhance productivity in bioreactors [67]. Solid phase immobilized biocatalysts have a number of advantages. A high concentration of catalyst can be maintained in the reactor for high volumetric productivity. Ease of catalyst retention allows repeated or extended use, and downstream processing is simplified. In addition, immobilization often has a

stabilizing effect [68]. Immobilized recombinant cells are being used increasingly. Plasmid stability in such systems is discussed below.

Effects of Immobilization

E. coli was the first immobilized recombinant organism studied. For this system, Inloes et al. [69] reported that a significantly higher cell concentration and consequently higher productivity of plasmid coded product could be obtained in a hollow fiber membrane bioreactor. Similarly, Dhulster et al. [70] immobilized recombinant *E. coli* BZ18/pTG 201 cells in κ -carrageenan beads, and obtained high cell densities in the cavities of the gel. Dhulster et al. [70] noted that immobilization might have an effect on plasmid stability. This effect was investigated by De Taxis du Poët et al. [71]. Plasmid-harboring *E. coli* (pTG201) was immobilized in κ -carrageenan, and plasmid maintenance was studied for both free and immobilized cells in a chemostat. Gel immobilization was seen to have a stabilizing effect; but once the cells were released from the beads, their plasmid-loss frequency was the same as for free cells. A theoretical analysis based on compartmentalization resulting from the immobilized growth was proposed [71]; but the plasmid stability predicted by this model was inferior to that actually observed. This suggested that factors other than immobilization may have contributed to the observed enhancement of stability. Later, De Taxis du Poët et al. [72] extended their analysis to recombinant *E. coli* JM105 containing the plasmid pKK223-200. Once again, immobilization enhanced plasmid stability. Increased plasmid stability was associated with the modified plasmid copy number, media used, oxygen limitation, as well as immobilization. Compartmentalization of cell growth, and growth gradients due to diffusional limitations in the immobilization matrix were said to explain the increased plasmid stability in immobilized cells. Stabilizing effect of immobilization has been observed also with *Bacillus subtilis* (pHV1431) [52].

Nasri et al. [73] examined the stability of pTG201 during continuous culture of three genetically different *E. coli* hosts. No selection pressures were employed, but the plasmid was stably maintained in all strains during immobilized culture. In contrast, in all strains, the plasmid showed various degrees of instability in continuous suspension

culture. Nasri et al. [73] concluded that increased plasmid stability was due neither to plasmid-transfer between immobilized cells, nor to an increase of the plasmid copy number in the cells. Two likely explanations for plasmid stability were offered: one was the absence of competition between plasmid-free and plasmid-containing cells in the immobilized system; and the second considered gel beads as a reservoir of plasmid-carrying cells. In further studies, the stability of three different plasmid vectors was examined in *E. coli* during immobilized continuous cultures [74]. In all cases, the loss of plasmids could be prevented by immobilizing plasmid-bearing cells in carrageenan beads.

Work by Sayadi et al. [50] showed that immobilization increased the stability of pTG201 considerably, even under conditions of high expression of the cloned product. A two-stage continuous immobilized cell system was described for maintaining high plasmid stability. In studies employing different environmental growth conditions, Sayadi et al. [75] reported that decreasing specific growth rate increased the plasmid (pTG201) copy number and the cloned enzyme's activity, but the stability decreased. Even under glucose, nitrogen, or phosphate limitation, immobilization enhanced the stability of plasmid [75]. However, with magnesium limited culture, the plasmids were relatively unstable, and the viable cell count declined during immobilized continuous culture [75]. These observations were not explained.

Detailed studies of plasmid stability in *E. coli* immobilized in κ -carrageenan gel beads were reported by Berry et al. [76]. Effects of factors such as inoculum size, gel bead volume, and gel concentration were examined. Plasmid (pTG201) stability increased with increasing inoculum size in the gel. Larger inoculum reduced the number of cell divisions required to fill the cavities in the immobilization matrix; hence, the culture time reduced, and the plasmid copy number remained high. In addition, because of the large inoculum, only few cavities were contaminated by plasmid-free cells, so there was little competition between plasmid-bearing and plasmid-free cells. Gel bead volume in the reactor, and the κ -carrageenan concentration in the gel apparently did not affect plasmid stability. The plasmid was extremely stable for the three bead volumes and three gel concentrations tested. Effects of agitation rate on plasmid stability in immobilized and free continuous

cultures of recombinant *E. coli* were examined by Huang et al. [77]. For free cells, the plasmid stability declined generally more rapidly when highly agitated; however, the gel immobilized recombinant cells displayed increased plasmid stability even when intensely agitated. The immobilization matrix must have limited the exposure of the cells to the agitated environment.

In a few studies, recombinant cells have been immobilized as biofilms. Huang et al. [78] reported on plasmid stability in suspension, and biofilm-immobilized cultures without selection pressures. In contrast to other data, the average probability of plasmid loss for suspended *E. coli* DH5a/pMJR1750 population was *lower* than that of biofilm-bound cells. No explanation was given, but biofilm immobilized cells probably did not experience the same confining environment as would occur in an entrapment matrix.

Information on immobilized culture of recombinant yeast is sparse. Continuous production of α -peptide using immobilized *S. cerevisiae* FY178 was reported by Sode et al. [79]. The peptide was secreted extracellularly. The cells were immobilized in Ca-alginate gel, and nonselective media were used for fermentation in a column reactor. Immobilization enhanced plasmid stability and α -peptide productivity. Walls and Gainer [80] described an immobilized *S. cerevisiae* Mc16 strain that contained the plasmid vector pMA230 (2 μ m based); the plasmid coded for amylase. The enzyme was secreted into the culture fluid. Glutaraldehyde was used to covalently couple the cells to gelatin beads. The beads were suspended in a fluidized bed bioreactor for continuous culture using a selective minimal medium. The immobilized cell system was examined at a dilution rate below washout to see if the attached population retained the plasmid while the free cells gradually lost plasmid. After 50 hours of continuous culture, the free cell population began to show plasmid loss, but the attached cells remained stable [80]. Furthermore, the plasmid stability in free cells varied with the dilution rate, greater plasmid loss was seen at lower dilution rates. No such variation occurred with attached cells [80]. This work was further extended to a different plasmid (p520, same yeast) that coded for a wheat amylase [81]. The immobilization method did not change, but a nonselective rich medium was employed. In this case, continuous suspension culture was unstable and rapidly lost the plasmid. The plasmid stability improved greatly upon immobilization, and a near constant

fraction of plasmid-containing cells was maintained during continuous culture. Relative to free suspension, immobilization improved protein productivity irrespective of the mode of operation of the reactor [81]. Several immobilization related effects—enhanced plasmid stability, increased cell concentration, and operation at high dilution rate—contributed to enhanced productivity.

In view of the many experimental observations, immobilization can be concluded to have a near universal stabilizing effect on recombinant cells. Several hypotheses have been proposed to explain this effect, but the specific mechanisms remain unclear.

Stabilizing Mechanisms

Stabilization that accompanies immobilization may have several possible sources, including one or more of the following: (i) immobilization may increase or maintain the plasmid copy number [50]; (ii) compartmentalization in the immobilizing gel may separate the plasmid-bearing cells from plasmid-free cells, thereby eliminating growth competition [71]; (iii) diffusional nutrient limitations in the gel may cause a growth rate gradient in the matrix, and hence morphological and physiological changes in immobilized cells [72]; (iv) the close proximity of immobilized cells could promote transfer of plasmid among them by either conjugation or transformation [78]; and (v) slower growth rate of immobilized cells may limit opportunities for plasmid loss [82]. These suggestions regarding possible mechanisms are largely speculative. A sound demonstration of the mechanism of stabilization at the molecular or cellular levels remains to be accomplished. In fact, enhanced plasmid retention upon immobilization may not have a single explanation; multiple interactions are more likely to be responsible.

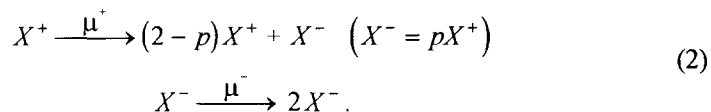
Our hypothesis on the stabilizing mechanism follows. Compared with freely suspended cells, immobilized cells are well known to have significantly altered growth rate (generation time), optimal growth conditions, and morphological forms [83]. These alterations presumably affect the cell cycle. In fact, there is some evidence that in immobilized cells budding may be delayed while decoupled DNA replication and polysaccharide synthesis continue [83]. In the immobilized recombinant cells, the delayed budding combined with continuing plasmid DNA replication may ensure that

offspring cells bear plasmids when the cell eventually divides. This situation would contribute to high plasmid copy number both in mother and daughter cells. A high copy number does not necessarily guarantee high segregational stability unless cells have efficient partition system; therefore, an improved partition system must be assumed for immobilized cells. Possibly the close contact among cells, and the confining effect of immobilization matrix, contribute to reducing or eliminating the partition bias of plasmids between mother and daughter cells. If immobilization results in high copy number and an improved partition system, the probability of plasmid loss would be reduced. Furthermore, immobilization may also affect the growth ratio. Because plasmid-containing cells are already metabolically burdened, and usually have reduced growth rates, further stress of immobilization may affect them less than plasmid-free cells. Hence, immobilization may reduce the growth ratio. Reduced growth ratio and probability of plasmid loss would ensure stability.

PLASMID INSTABILITY KINETICS

Models of plasmid instability can provide valuable information about the nature of instability, thus helping to improve the understanding of this complex phenomenon. Models may allow predictions of the extent of plasmid stability. Such information is potentially useful in determining optimal bioreactor operations for maximizing the yield of the recombinant product. A large number of models of plasmid stability have been developed [62, 84–90]. Mostly these models depend on two important kinetic parameters: the probability of plasmid-loss (p) due to structural instability or/and segregational instability, and the growth ratio (α) reflecting the specific growth rate difference between plasmid-bearing and plasmid-free cells.

In analyzing plasmid loss in batch culture, Imanaka and Aiba [61] represented a mixed population of plasmid-carrying (X^+) and plasmid-free (X^-) cells as follows



Imanaka and Aiba [61] further assumed: (i) cells that lose plasmids cannot regain them; (ii) host cells growth is exponential; (iii) a constant probability of plasmid loss

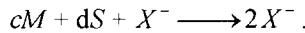
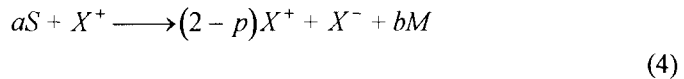
per cell division; (iv) a constant growth ratio; and (v) zero initial concentration of plasmid-free cells. According to the model, the plasmid-carrying cell fraction would decline monotonically with generation number; thus,

$$F_n = \frac{1 - \alpha - p}{1 - \alpha - p \cdot 2^{n(\alpha + p - 1)}}, \quad (3)$$

where $\alpha = \mu^-/\mu^+$. From equation (3) it is obvious that in batch systems with any $\alpha > 1.0$ and $p > 0$, $F_\infty = 0$. The Imanaka and Aiba [61] model is unstructured, but elegantly simple. In principle, any recombinant organism satisfying the underlying assumptions should conform to the model. Unfortunately, the p and α usually vary during the culture period, and the assumed exponential growth is not exactly followed.

Pioneering developments in structured modeling of mixed recombinant culture came from Bailey's group working with recombinant *E. coli* [84, 85]. The plasmid stability and the expression of a cloned-gene product were described by a model based upon the molecular mechanism of plasmid replication, partition, and transcription. Later, at the single-cell level, using population balance models, Hjortso and Bailey [57, 91] described plasmid stability in the yeast *S. cerevisiae* with and without selection pressure. Two assumptions were examined for plasmid partitioning: (i) random and independent distribution of plasmids between the mother and the daughter cells at partition; and (ii) a greater or equal probability of a plasmid residing in the mother cell after division than in the daughter cells. Two models were developed based on the two mechanisms for plasmid replication. In the first model, the cells replicated plasmids such that the total number of plasmids was the same for all dividing cells in the population. In the second, the cells produced plasmids at the same rate, irrespective of the initial copy number. The results obtained from those models were compared with the corresponding results from a nonstructured model, and pronounced differences were found at low growth rates. These models were able to relate observable bulk properties of growth to kinetic events at the cell level. In later work, a general single-cell model for plasmid propagation in recombinant yeast was developed [88]. This model included the plasmid burden effects on host cell growth rate, and it could be combined with bioreactor performance equations to simulate a production process.

A macroscopic population dynamics model for plasmid stability in continuous culture of recombinant *S. cerevisiae* with selection pressure was described by Sardonini and DiBiasio [59]. Several assumptions were made, including: (i) Plasmid-free cells could propagate to some degree in the selective medium. [This growth could be explained by a metabolite being excreted into the medium by the plasmid-carrying strain. This metabolite supported the growth of plasmid-free cells.] (ii) A constant probability of plasmid loss. (iii) A single substrate (S) limits growth of plasmid-carrying cells in accord with Monod kinetics. (iv) Growth rate of the plasmid-free cells is limited by this substrate (S) as well as an additional metabolite (M) according to a dual Monod form. (v) Continuous culture with a constant reactor volume, and sterile feed. The fermentation process could be represented as



A chemostat mixed culture could be expressed [59] with the equations:

$$\frac{dX^+}{dt} = (1 - p)X^+\mu^+(S) - DX^+ , \quad (5)$$

$$\frac{dX^-}{dt} = p\mu^+X^+ + \mu^-(S, M)X^- - DX^- , \quad (6)$$

$$\frac{dS}{dt} = D(S_0 - S) - \frac{\mu^+(S)X^+}{Y_s} - \frac{\mu^-(S, M)X^-}{Y_s} , \quad (7)$$

and

$$\frac{dM}{dt} = k\mu^+(S)X^+ - \frac{\mu^-(S, M)X^-}{Y_m} - DM , \quad (8)$$

where

$$\mu^+(S) = \frac{\mu_m^+ S}{K_s + S}, \quad (9)$$

and

$$\mu^-(S, M) = \mu_m^- \frac{S}{K_s + S} \frac{M}{K_m + M}. \quad (10)$$

Mixed cultures exhibit multiple steady states, and the conditions for these states can be determined by setting equations (5)–(8) equal to zero. Because of the dependence of X^- cell growth on the presence of X^+ cells, washout of any one strain while retaining the other is impossible. Thus, in a single chemostat mixed culture in selective media, only two possible steady can exist: either total washout ($X^+ = 0, X^- = 0$), or coexistence ($X^+ > 0, X^- > 0$). The Sardonini and DiBiasio [59] model allows for plasmid instability due to growth of plasmid-free cells in spite of selection pressure, and that due to plasmid loss. Moreover, the model directly relates to measurable macroscopic parameters. The results predicted by this model agree with experimental data. Furthermore, the model shows that in chemostat mixed culture without selection pressure, coexistence is impossible if $\mu^- > \mu^+$, and $p > 0$. If this occurs, the original plasmid-bearing population will be eventually replaced by plasmid-free cells.

Despite advances, mechanisms of yeast plasmid instability are not fully understood; hence, models of plasmid instability are often substantially based on conjecture. Modeling of immobilized recombinant cell systems remains to be addressed. Approaches for improving plasmid stability are summarized in Table 2.

CONCLUDING REMARKS

Here we focused on plasmid stability in recombinant *S. cerevisiae*. Stability considerations were discussed at molecular, cellular, and engineering levels. In addition, the environmental factors that affect stability were examined. With improved understanding, plasmid stability may be enhanced by manipulating plasmid composition and structure, modifying the genetic and physiological properties of host

Table 2. Strategies for enhancing plasmid stability in recombinant yeast.

Genetic Approaches	Environmental Approaches
Stabilizing genes	Addition of antibiotics
Efficient partition genes	Amino acids deficient media
Nature fitness genes	Optimum dissolved oxygen tension
Efficient replicating origins	Temperature shift
Optimal copy number	Cyclic changes in dissolved oxygen tension
Regulation of gene expression	Dilution rate oscillations
Suitable promoter strength	Oscillation of substrate concentration
Antibiotic resistance markers	Fed-batch culture
Copper resistance markers	Two-stage continuous culture
Auxotrophic mutants	Selective cell recycle
Autoselection systems	Immobilized systems
Killer toxin-immunity strains	

cells, and controlling the environment. At the molecular level, improvements in stability of plasmid constructs are already being achieved through insertion of particular DNA sequences that alter replication and partitioning behavior, as well as by modifications of the genotype of the host. Yet more stable plasmids will certainly emerge in view of the massive theoretical and experimental effort being devoted to this area.

Although the molecular details of plasmid constructs and biology of the cell are of interest, biochemical engineers are concerned primarily with maintaining plasmid stability through environmental manipulations including media composition and selection pressures, dissolved oxygen, temperature, pH, and the mode of cultivation. Of the many strategies proposed to overcome instability, only selective media and

autotrophic strains are used most commonly. So far no other effective methods exist for use with large scale culture. Nevertheless, highly defined selective media are expensive for commercial applications; hence, manipulation of other environmental variables to achieve stability in nonselective media remains a major objective. To what extent, if at all, specific environmental variables affect stability cannot at present be predicted a priori.

Immobilization does afford high cell concentrations and productivity of the gene product, as well as plasmid stability over longer periods relative to free cell systems. Therefore, immobilization may become a preferred culture technique for recombinants in nonselective media. But little is known about immobilized recombinant yeast, especially about the mechanisms that enhance plasmid stability. Moreover, logistics of large scale immobilized culture, and some of its well known problems [3], need addressing if this method is to gain acceptance. Work is needed also on any genetic, physiological, and morphological changes that accompany immobilization, as well as on models of immobilized systems. All those unknowns provide opportunities for breakthroughs that are likely to contribute to establishing commercially useful immobilized recombinant systems.

NOMENCLATURE

a	Stoichiometric coefficient
b	Stoichiometric coefficient
c	Stoichiometric coefficient
d	Stoichiometric coefficient
D	Dilution rate
F_n	Fraction of plasmid-bearing cells after n generations
F_∞	Fraction of plasmid-bearing cells after infinite generations
k	Growth associated coefficient for metabolite formation
K_m	Monod constant for metabolite
K_s	Monod constant for substrate
M	Concentration of plasmid-coded metabolite
n	Cell generation number

p	Probability of plasmid loss
S_0	Initial substrate concentration
S	Substrate concentration
X^+	Concentration of plasmid-bearing cells
X^-	Concentration of plasmid-free cells
Y_m	Yield coefficient for metabolite
Y_s	Yield coefficient on substrate
α	Growth ratio
μ^+	Specific growth rate of plasmid-bearing cells
μ^-	Specific growth rate of plasmid-free cells
μ_m^+	Maximum specific growth rate of plasmid-containing cells
μ_m^-	Maximum specific growth rate of plasmid-free cells

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