reviews

Strategies for improving plasmid stability in genetically modified bacteria in bioreactors

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Exploitation of recombinant organisms for the large-scale, commercial production of foreign proteins is often hampered by the problem of plasmid instability. A wide range of strategies have been reported for improving the stability of recombinant organisms. A combination of manipulating both the genetic design of recombinants and the conditions of culturing the organisms may be used to achieve stable host–vector associations during culture of recombinant organisms in bioreactors.

The advent of recombinant-DNA (rDNA) technology has provided a direct approach for the production of a wide range of biochemical products from industrial microorganisms, as well as from mammalian and plant cells. It has also provided the means to improve the economics of the bioprocess, if and when properly implemented.

The organisms most commonly exploited for industrial production purposes are *Escherichia coli*, *Bacillus subtilis* and the yeast *Saccharomyces cerevisiae*, and they are used primarily for the production of recombinant proteins. To manufacture such proteins, DNA sequences encoding the protein must be transferred into the cells. This is usually achieved by the introduction of extrachromosomal DNA in the form of plasmids or phages. The cells which carry these recombinant genes can be cultured in special bioreactors for the large-scale production of proteins including a high proportion of specific product.

The second most common application of these microorganisms is the introduction of new enzymatic activities, for example, to modify the metabolic pathways in the cells. These modifications may be used to generate greater quantities of enzymes, to deregulate existing metabolic pathways or to create new ones. This technique, termed 'metabolic design', is used to produce higher levels of amino acids and to expand the substrate spectrum of microorganisms. The creation of such modified systems is achieved by sequential steps, including identification and isolation of the required genes, construction of the expression vectors and screening for the optimal host system. Generating the genetic equipment in a desired host is followed by the optimization of the bioprocess, which involves scale-up and the technology for the separation of the product.

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Plasmids as vectors

The use of plasmids as vectors is almost indispensable in genetic engineering for the expression of foreign genes in prokaryotic (bacteria) or eukaryotic (yeast) cells. Plasmids are non-essential extrachromosomal DNA elements. They replicate autonomously and are inherited with different rates of fidelity, but are not essential for cell viability. Although a variety of plasmids are known and they are classified into different groups[1], not all are of use in commercial bioprocessing. Desirable characteristics for useful vectors include (1) high copy number, (2) possession of several unique restriction endonuclease cleavage sites, (3) small size, (4) genetic stability, (5) screening markers (e.g. antibiotic resistance gene encoded by the plasmid), and (6) simple procedures for transfer into the host. High plasmid copy number leads to higher concentration of transcription template. To use any plasmid as a vector it should bear several restriction sites for inserting DNA fragments. A small plasmid imposes less of a metabolic burden on the host and also facilitates transformation. However, probably the most important parameter is that the expression plasmid should be stably maintained in the host for several generations.

The productivity of a bioreactor employing recombinant strains is largely affected by the degree to which the plasmid-free (P^-) cells are generated and propagated. This phenomena was observed by many workers and is responsible for further complications in the scale-up which is required for commercializing the recombinant products[2–5]. The P^- cells are generated from plasmid-harboring

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(P^+) cells by segregational instability which is caused by defective partitioning of the plasmids between the daughter cells during cell division[6]. Another source of instability originates from changes in the plasmid itself such as point mutation, deletion, insertion and rearrangement in the plasmid DNA[7,8]. The resulting cells (with plasmid absent or structurally altered) are non-productive.

The P^+ cells usually grow more slowly than the P^- cells because the P^+ cells have to synthesize more DNA, mRNA and protein[9]. This leads to a lower maximum growth rate (μ_{max}) for P^+ cells[3,9,10]. In addition, the growth rate of P^+ cells also depends upon the toxicity of the coded proteins[11] and strength of the promoters. Thus, once generated in the reactor, P^- cells may propagate rapidly, leading to a mixed population with the P^- proportion of population increasing with each generation, leading, in turn, to poor economics of the total bioprocess[2,3,5].

Although continuous systems are highly productive for many microbial production processes, their application is more limited for recombinant organisms because of plasmid instability. Most recombinant proteins are therefore produced by either batch or fed-batch techniques. The generation of P^- cells is a common phenomenon in both continuous and batch culture. However, the situation is more complicated in continuous processes where the cells go through a greater number of generations than in batch processes. The consequences will be more serious in continuous cultures when the P^- cells have a growth advantage together with high segregational instability. Therefore, it is a two-faceted problem, where the P^- cells should be eliminated or destroyed or inhibited to allow further proliferation in the medium and simultaneously intrinsic plasmid stability should be improved.

Improving plasmid stability

To achieve this, a few strategies have been proposed and these are broadly categorized by Ensley[2] into selective and non-selective methods.

Selective methods

The selective methods include maintaining selection for antibiotic resistance by use of antibiotics in the growth medium, complementation of host auxotrophy by incorporating auxotrophic markers on plasmid vectors, lysogenic phage repression and incorporation of suicide proteins and RNA whose synthesis is repressed in the presence of the plasmid. (The mechanism of stabilization by the expression of suicide proteins from plasmid-free cells is described in Ref. 12.)

Non-selective methods

Non-selective methods include incorporation of partition loci to obtain controlled partitioning of the plasmid to the daughter cells during cell division, compensation of auxotrophic defect of the host coded on the plasmid and also application of specific culture conditions.

Various aspects of plasmid stability in recombinant cultures have been reviewed: these include the use of enzyme systems to measure plasmid stability[3], the effect of copy number and cultivation strategies[5], mathematical modelling of plasmid stability[9], control of plasmid replication[13], plasmid partition[14], and plasmid stability in cell immobilization[15]. This review focuses on some bioprocess strategies and presents an alternative classification of strategies for improving the stability of genetically modified bacteria.

Strategies to stabilize cultured recombinant organisms

Plasmid stability is obviously an essential prerequisite for successful expression of heterologous proteins. To achieve this, a number of strategies for improving stability of plasmids have been reported (Table 1).

We classified these strategies into either cellular/molecular or bioprocess strategies based on their principles. The cellular/molecular strategies include, for example, modulating genes at the segregational step and post-segregational effects. These methods may prevent the formation of P^- cells in the reactor (*par*A locus[14] and *cer* function[16,17]), or kill or inhibit proliferation of the P^- cells after the segregational step (λ lysogens[18], *ccd* function[12,19,20], streptomycin dependency[21], bacteriocins[22], *ssb* gene[23], *val*S[24], antibiotic supplement, *asd*$^+$ system[25], *dal* functions[26], employing either single[27] or double auxotrophic mutants[28]). Chromosomal integration is another strategy[29]. Bioprocess strategies also include inhibition or separation of P^- cells from the mixed population in a bioreactor. Only a few strategies such as two-stage cultivation[30], recycling conditions (between different dilution rates[31] or different substrate concentrations[32]), were shown to nullify the growth advantage of P^- cells. Recently, separation of P^- cells from the mixed population has been reported using selective flocculation[33], and also attempted using aqueous two-phase system[34]. Application of the technique of whole-cell immobilization to recombinant organisms was also found to improve the stability of plasmids and expression of the encoded gene products[35,36].

Strategies (reported so far) are designed mainly to overcome the segregational instability of plasmids and to nullify or eliminate the growth advantage of P^- cells. In some cases structural instability of the plasmids have also been observed to occur at high frequency during cell culture. (Cells carrying such unstable, altered plasmids are termed P*.) Their existence may not be readily apparent during cell culture because they usually have the same specific growth rates as cells bearing unaltered plasmids: their detection is difficult because small rearrange-

ments in the plasmid DNA do not always lead to the inactivation of marker genes (e.g. antibiotic resistance genes) which are detectable by selective plating. However, the frequency of occurrence of P* cells was reported to be very low with many plasmids. Presumably, this is mediated by a transposable element in the host JM103. The expression of the *xylE* gene is found to be five- to sevenfold lower when compared with the original plasmid (unpublished observations). Therefore, we believe that it is important to know the frequency of their occurrence in order to improve the productivity of the bioprocesses. Nevertheless, there is as yet no strategy available to eliminate the structural instability of the plasmids except that of employing a mutation deficient (*rec*$^-$) host.

Cell internal factors influencing stability of recombinants

The presence and transcription of specific sequences located on plasmid expression vectors can lead to plasmid instability. For instance, Chiang and Bremer[37] studied the stability of plasmid pBR322 and its *rom*, *bla* and *tet* derivatives†: they suspected that the transcription of the *tet* gene affects cell viability and might also contribute towards reduced plasmid stability. This was confirmed by identifying a region within the *tet* promoter of pBR322 whose deletion leads to an increase of stability of the plasmid derivative. They demonstrated that the Δrom and Δbla derivatives were lost from the host within 60 generations, while the Δtet derivative was stable under similar conditions (NB Δ = deletion of all, or part, of the gene sequence on the plasmid). It should be noted that there may exist such important sequences which affect stability in other plasmids.

Both the size of the inserted DNA and the act of introducing foreign DNA are factors which can affect genetic stability. Warnes and Stephenson[38] reported that insertion of foreign DNA up to 2 kb in size had no effect on plasmid stability, though the stability started to be affected when the inserted fragment length was increased to 8 kb, and was severely affected by a 21 kb DNA insert. The loss of plasmid may be due to problems with replication fidelity, segregation or low copy number.

Earlier, it was proposed that 'illegitimate' recombination might be a consequence of errors of the DNA-modifying enzymes during rearrangement or replication procedures, which affect stability of the plasmids[39]. Smaller size plasmids (<10 kb), used as vectors for Gram-positive bacteria, are replicated by the 'rolling-circle replication'. By this mode of replication single-stranded DNA (ssDNA) is generated and such plasmids are therefore known as ssDNA plasmids. These plasmids commit frequent errors during replication. In some Gram-positive bacteria the plasmids replicate (size >10 kb) with low error levels. These plasmids use the mechanism of 'theta replication' instead of 'rolling-circle', suggesting that the former replication might be less error-prone than the latter one. Using these plasmids, large DNA fragments (up to 40 kb) can be efficiently cloned and maintained for 150 generations. Other systems need to be studied, however, to examine the generality of this observation.

In view of the host physiology, it might be helpful to use regulatory promoters to improve the stability problem. Plasmids which carry such promoters may eliminate or reduce the expression of cloned gene or toxic gene product during growth phase, by which the growth advantage of P$^-$ cells can be avoided. This is particularly significant when there exist high difference growth rates between the P$^+$ and P$^-$ cells. To date, a few regulatory promoters are known for their strict on/off control of the cloned gene expression.

Table 1. Various strategies for improving plasmid stability or for overcoming the growth advantage of P$^-$ cells in genetically modified organisms

Strategies	Method	Refs
Cellular/Molecular strategies		
Modulating genes for stable maintenance during segregational step	*par*A loci	14
	cer function	16, 17
Post-segregational killing or inhibiting functions/compounds	λ lysogens	18
	ccd functions:	
	F plasmids	19
	R 100	20
	R1 (Hok/SoK protein)	12
	Streptomycin dependency	21
	Antibiotic addition to medium[a]	
	Bacteriocins	22
	ssb gene	23
	*val*S system	24
	asd$^+$ system	25
	dal functions employing amino acids	25
	Auxotrophic mutants:	
	Single	27
	Double	28
Other strategies	λ vectors: (chromosomal integration)	29
Bioprocess strategies		
Inhibition of growth advantage of P$^-$ cells	Two-stage cultivation	30
	Recycling between:	
	different dilution rates	31
	different substrate concentration	32
Separation of P$^-$ cells from P$^+$ cells	Selective flocculation	33
	Aqueous two-phase system	34
Other strategies	Whole-cell immobilization:	
	E. coli	35
	B. subtilis	36

[a] General method.

† NB *rom* = *rop*, repression of primer (control of copy number of colE1 plasmids); *bla* encodes β-lactamase; and *tet* encodes tetracycline resistance. Nomenclature is taken from Ref. 37. The plasmid pBR322 encodes ampicillin (Ap) and tetracycline (Tc) resistance.

Culture conditions influence recombinant stability

Optimizing environmental conditions for the culture of organisms is an important consideration for successful operation of any bioprocess. However, for culturing recombinants, there is the additional factor to be considered – namely, plasmid stability. Therefore, in bioprocesses using recombinant organisms, the objectives are high plasmid stability, high volumetric productivity, high yield coefficients and low costs of ingredients and energy. For recombinants, plasmid stability during cell culture is a primary consideration, with other factors which contribute to improved productivity of secondary importance. Under normal operating conditions the relative density of P^- cells in the reactor increases due to greater segregational instability or growth-rate differences between host P^- and P* cells. Nutrient limitation places relatively greater stress on the P^+ cells, leading to an increase in the proportion of P^- cells in the reactor population. Several reports indicate that limitation of carbon, nitrogen, phosphate and minerals can be detrimental to plasmid stability. The *E. coli* K12 strain bearing the plasmid TP120 (carrying ampicillin-, streptomycin-, sulfonamide- and tetracycline-resistance genes) was grown under carbon- and phosphate-limiting conditions, leading to the loss of the tetracycline-resistance gene[8]. On the other hand, for the same plasmid ampicillin- and sulfonamide-resistance genes were lost only under phosphate limitation[8]. Similar observations were made in the same host with the plasmid pBR322, where the *tet* and *bla* genes are lost after prolonged culture under glucose- and phosphate-limiting conditions in continuous process at lower dilution rates[40]. There are reports on the loss of *tet* gene from the plasmid pBR325 under glucose- and nitrogen-limiting conditions at lower dilution rates[41]. The stability of the plasmid pTG201 (carrying *xyl*E gene) was strongly affected by the depletion of glucose and ammonia. Similar instability was also observed in a Mg^{2+}-deficient medium[42]. The reason for such instability could be the reduction of the plasmid copy number in nutrient-limited conditions. The plasmid copy number is particularly affected by phosphate and magnesium limitation in M9 minimal medium while glucose and ammonia limitation leads to a (50–100%) increase of the plasmid copy number[42].

The plasmid pBR325 in host *E. coli* GY2354 lost all resistance characters (Ap, Tc and Cm) at 0.15 h^{-1} dilution rate in glucose limited conditions. (NB Ap = β-lactamase [cleavage of β-lactam ring]; Tc = tetracycline resistance gene [399 amino acid peptide inhibits tetracycline incorporation]; Cm = chloramphenicol resistance [acetylation of chloramphenicol].) However, pBR325 in a different host, *E. coli* GM31, under similar conditions, also lost all the resistance characters, though the rate of loss of tet^R was faster compared with the rate of loss in *E. coli* GY2354[43]. This demonstrates clearly the effect of host identity on plasmid stability. The problem is more complex, however, than just a question of which host to use: pHSG415 in the *E. coli* RV308 host was found to be unstable during a non-limited batch culture, but to maintain stably in continuous culture under phosphate- and nitrogen-limiting conditions[43]. In general, use of a complex growth medium (Luria broth, LB, medium) has been found to stabilize some plasmids during cultivation.

Recombinant microorganisms usually accumulate high intracellular concentrations of the proteins expressed from the introduced genes. In addition, they are grown to high cell densities, essential for increasing productivity in bioprocesses. For large-scale culture in particular, this leads to the need for an inexpensive, simple C-source substrate such as glucose. Over-feeding with glucose, however, leads to a bacterial 'Crabtree effect'‡ under aerobic conditions in which acetate and CO_2 formation takes place[44]: high levels of these metabolites in the culture broth can inhibit further growth. To overcome these limitations, a fed-batch culture strategy using different feeding policies has been developed for *E. coli* and *Saccharomyces cerevisiae*[45–48]. Acetate production depends strongly upon the carbon and salt concentration, and the growth rate. It was suggested that high dilution rates result in accumulation of acetate due to saturation of the respiratory system[45]. The fed-batch process for the culturing *E. coli* was found to avoid acetate formation, with different feeding policies being used to obtain high cell density cultures of more than 100 g L^{-1}. Zabriskie *et al.*[46] exploited a fed-batch technique in order to achieve high cell densities along with high level expression of the malaria antigen in *E. coli*. They showed that, using different carbon-feeding policies it was possible to control effectively the specific growth rate prior to induction without a reduction in the expression level (i.e. with the stable maintenance of the expression plasmid). Tsai *et al.*[47] used two kinds of fed-batch processes (different in feed medium composition) for the production of human-insulin-like growth factor (IGF-1) in *E. coli*. The dual feed process (feeding of organic nitrogen source at 50 g h^{-1}) was found to improve the yield of IGF-1 by a factor of ten. Recently, Horn *et al.*[48] reported that using a fed-batch process with different feed policies, the segregational and structural stability of plasmids (pUR290 and pFB99) was not affected. They also found low levels of acetate during culture and achieved high cell densities (45–50 g dry weight L^{-1}).

The effects of temperature on the production of recombinant proteins need further investigation,

‡ Crabtree effect – the inhibition of oxygen consumption in cellular respiration that is produced by increasing concentrations of glucose.

since plasmid stability and copy number may sometimes be affected. The plasmid pLP11 in host *Bacillus stearothermophilus* was found to be stable below 50°C, but above this temperature the plasmid stability progressively decreased[49]. Son *et al.*[50] demonstrated the effects of temperature on plasmid stability and expression of a cloned gene (cellulase) in a recombinant *Bacillus* strain. Although both high plasmid copy number and efficient gene expression are favoured by higher temperatures, the plasmid was stably maintained only at temperatures below 30°C in batch culture.

A particular problem for scaling-up bioprocesses is the construction of reactors which allow a high level of oxygen input and in which the medium is well mixed. This problem is exacerbated by increases in cell density. The dissolved-oxygen tension in the medium is well known to have significant effects on the metabolism of microorganisms. Hopkins *et al.*[51] investigated the effect of a dissolved-oxygen shock on the stability of the plasmid pKN401 in *E. coli*. They showed that the plasmid was stable in either non-selective or selective conditions during culture, but applying a shift to zero in the dissolved oxygen (i.e. oxygen starvation) for a short period (oxygen shock) the system shows a strong segregational instability. Ryan *et al.*[52] showed that the aeration rate was detrimental to the cell growth but beneficial for the β-lactamase expression. Caunt *et al.*[53] reported similar results on the effect of oxygen limitation on the plasmid pLG669-2. The proportion of plasmid-bearing cells decreased more rapidly at lower concentrations of dissolved oxygen. An increased rate of loss of the plasmid appeared to be the major cause for the instability. However, under oxygen limitation, several host–plasmid systems are considerably less stable than under unlimited conditions[51]. Thus, micromixing problems in industrial scale bioreactors need to be solved for the culture of recombinant systems.

A number of strategies to overcome the instability problem of expression plasmids have been described in this review. Combining some of these strategies together with appropriate genetic and environmental conditions for the host, may lead to host–vector interactions compatible with plasmid stability under non-selective conditions and, thus, to the successful exploitation of recombinant organisms in bioreactors.

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