Recombinant DNA technology has not only provided a direct approach for the production of a wide range of therapeutic proteins from microorganisms but it has also provided the means to improve productivities of bioprocesses. Among the organisms available for the commercial production of recombinant proteins the most commonly exploited are, *Escherichia coli* and *Bacillus subtilis.*

An important consideration in the scale-up of fermentations involving recombinant microorganisms is the stability of the plasmid employed during the entire course of production. Although a number of studies have been carried out analyzing the causes of plasmid instabilities in various organisms, there is little information on their comparative stabilities in different host backgrounds.

In the present thesis an attempt to examine the causes of plasmid instabilities in *E. coli* and *B. subtilis* hosts by the use of shuttle vectors capable of replication in either host, has been made. In addition, studies on various bioprocess strategies to minimize plasmid loss during fermentation have also been carried out. The bifunctional shuttle plasmid, pUSH2, has been used as a model system to study the kinetics of plasmid loss in either host. Attempts to alleviate plasmid loss during extended fedbatch fermentations by the use of different feeding strategies have been the focus of the present study.

Fermentations carried out using the shuttle plasmid, pUSH2, showed the plasmid to be segregationally more unstable in *E. coli* than in *B. subtilis*. Segregational stability was estimated by determining the fraction of the cells harbouring the plasmid during the course of fermentation on antibiotic selective plates. No structural instabilities in the plasmid were detected in either host. Batch fermentation experiments conducted using various media in the order of increasing complexity showed that the plasmid stability in *E. coli* decreased with increasing medium complexity and specific growth rate. To test the hypothesis that higher growth rates resulted in greater plasmid instabilities, fermentations were carried out at different temperatures ranging from 25^0C to 40^0C. Temperature effects on cultivation conditions showed the plasmid to be unstable at higher temperatures where specific growth rates were correspondingly higher, and

relatively stable at lower temperatures where specific growth rates were comparatively lower. Experiments on the effects of levels of dissolved oxygen concentration in the bioreactor showed that lower dissolved oxygen concentrations in the fermenter resulted in a drastic drop in plasmid stabilities at low specific growth rates, in the case of *E. coli*, but not in the case of *B. subtilis*. This indicated that plasmid loss could be host related. Fedbatch fermentations to increase biomass yield and productivities were carried out using various feeding profiles *viz.*, constant feeding, exponential feeding, increasing rate feeding, and decreasing rate feeding. Results of these fermentations showed that although the exponential feeding strategy carried out with an objective of maintaining a constant specific growth rate of 0.17 h^{-1}, yielded the highest cell density of 13.2 g/l, the decreasing feed strategy seemed to improve plasmid stabilities the most. A final plasmid stability of 79% was achieved in the case of decreasing rate feeding as compared to 53% in the case of exponential feeding. The pulse feeding strategy based on feeding nutrients corresponding to the levels of dissolved oxygen concentration in the fermenter, destabilized the plasmid to the highest extent in *E. coli*. The plasmid stability dropped to 31% as compared to other feeding strategies where it ranged from 53% to 79%. Continuous fermentations at a constant growth rate showed a steady decline in plasmid stability in *E. coli* resulting in a final plasmid stability of 69%. The plasmid showed very high stabilities (>95%) in *B. subtilis* during all modes of fermentation.

The effects of plasmid size and metabolic load on the host cell were also studied using the shuttle plasmid, $pVSH_2GA$, constructed by the fusion of the two medium sized plasmids pUSH2 and pMLB1010. This plasmid coded for ampicillin resistance and β-galactosidase in addition to the kanamycin and chloramphenicol resistances displayed by pUSH2.

$pVSH_2GA$ was found to be segregationally unstable to a greater extent than pUSH2 in both *E. coli* and *B. subtilis*. The specific growth rate advantage of the plasmid-free *E. coli* host was much larger in the case of *E. coli* harbouring $pVSH_2GA$ as compared to *E. coli* harbouring pUSH2, signifying the effects of a much larger metabolic load due

to $pVSH_2GA$ on the host cell. A similar large growth rate difference in the case of *B. subtilis* was not observed and this corresponded well with the fact that most of the genes expressing in *E. coli* were not expressed in *B. subtilis*. The use of complex media, yielding high specific growth rates, during batch fermentations of *E. coli* harbouring $pVSH_2GA$, resulted in high plasmid stabilities and β-galactosidase activities as compared to fermentations carried out in minimal M9 media supplemented with either glucose or glycerol. Plasmid stabilities in the case of *E. coli* grown in the presence of lactose as the sole carbon source were higher (75%) even at low specific growth rates probably because only those cells that could utilize lactose survived. Plasmid stabilities dropped to 90% in all cases in the case of *B. subtilis*. Fermentation experiments carried out at various dissolved oxygen concentrations ranging from 0% saturation to 100% saturation resulted in similar trends as seen in the case of the plasmid pUSH2. β-galactosidase activities improved with an increase in dissolved oxygen levels in the fermenter upto 50% dissolved oxygen saturation, and thereafter reached a maxima.

Fedbatch fermentations were carried out using similar feeding techniques as used for the strains harbouring the plasmid,pUSH2. In all cases of feeding, there was a steady drop in specific growth rate profiles and rates of plasmid loss were directly related to the rate of drop of specific growth rates. A higher drop resulted in a higher plasmid loss rate. Plasmid stabilities were >90% in all cases in the case of *B. subtilis* harbouring $pVSH_2GA$. The decreasing rate feeding profile alleviated the rate of drop in specific growth rates and thus stabilized the plasmid the most. In *B. subtilis* also the decreasing rate feeding improved plasmid instabilities by 5%. Lactose feeding improved β-galactosidase activities in *E. coli* although biomass concentrations achieved were quite low. Highest β-galactosidase activities were also observed during the decreasing rate feeding strategy. The instability of $pVSH_2GA$ as compared to pUSH2 was marginal in *B. subtilis* with a drop in stability of ~8%, while the corresponding drop in *E. coli* was ~40%. No structural instability of the plasmid, $pVSH_2GA$, was observed in either *E. coli* or *B. subtilis* hosts.

Fermentations carried out using the larger plasmid, $pVSH_2GA$, showed higher instabilities compared to the smaller plasmid, pUSH2, under similar fermentation conditions and the instabilities could be stabilized to a significant extent by similar bioprocess strategies.

In conclusion it can be said that recombinant plasmid instabilities are host-vector specific in most cases and to a large extent depend on the metabolic load imposed by the vector on the host. The plasmids pUSH2 and $pVSH_2GA$, are maintained differently in different host backgrounds. However, their stability characteristics could be further improved using similar bioprocess techniques employed in this study to enhance product yields.