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Short communication

Model-based evaluation of plasmid segregational instability in repeated batch culture with recombinant *Escherichia coli*

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ABSTRACT

Technological advancement in molecular biology techniques has proved to be useful in successful overexpression of recombinant proteins. However, one of the major issues of concern is the instability of recombinant plasmid when the recombinant hosts are grown without selection pressure. Current study proposes a correlation between segregational instability and plasmid size. In order to explain the segregational instability, existing mathematical model have been used. Four different recombinant vectors pET29b, pET32b, pET43b and pCS22, all having 1.5 kb α-amylase gene (*amyE*) insert from *Bacillus amyloliquefaciens* were used in the study. Culture when grown in repeated batch without suitable selection pressure showed plasmid free cells generating from plasmid bearing cells. Segregational instability of 2.2×10^{-3} and 1.4×10^{-3} per generation was observed with pET43b-*amyE* plasmid and pCS22-*amyE* plasmid respectively. This is the first report on segregational instability of commercially available vectors. A mathematical correlation has been proposed between plasmid size and rate of segregational loss per generation (R).

1. Introduction

E. coli has been the workhorse for the production of various recombinant products that are commercially important. Many different expression vectors are available for efficient production of recombinant proteins and they differ from one another in various vector characteristics such as size, promoter, fusion tag and selection marker. Often, the recombinant system encounters certain issues that make the overall process of protein production uneconomical. One such issue of major concern is plasmid instability [1]. Plasmid instability can be either structural instability or segregational instability [2]. Structural instability can arise due to deletion, insertion, point mutation or the involvement of transposons and is not very common. Previous studies using mutant host (*rec*$^-$) have shown success in improving structural instability of some such vectors [3].

1.1. Plasmid segregational instability

The major cause of plasmid loss from a growing recombinant cell population is segregational instability [4]. Plasmid instability or plasmid loss is the result of additional metabolic loads imparted by the vectors on host cells. High copy number plasmid vectors are segregated among daughter cells by random segregational mechanism during cell division and resulting daughter cells may receive unequal number of vectors from the parent cell [5]. The presence of an external selection pressure, such as use of antibiotics, permits the growth of plasmid bearing cells only and thereby maintains homogeneity of the recombinant cell population [2]. However, the use of antibiotic is not usually feasible for two reasons: cost of antibiotics and regulatory issues regarding contamination of final product [6]. In the absence of any external selection pressure, plasmids may dilute out from the daughter cells in subsequent generations leading to a heterogeneous population of plasmid bearing and plasmid free cells.

1.2. Mathematical model for plasmid instability

Different mathematical models and probability based approaches were designed to explain plasmid instability in recombinant cell population [7–11]. However, some of them are based on such experimental parameters which are often difficult to analyze experimentally [9]. Current study focuses on the segregational instability of some of the commonly used expression vectors in repeated batch. Results were interpreted based on mathematical models proposed by Cooper et al. [7] and Imanaka and Aiba [8].

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2. Materials and methods

2.1. Bacterial strains and plasmid

The strain *Bacillus amyloliquefaciens* MTCC 610 was obtained from Microbial Type Culture Collection, Institute of Microbial Technology, India (Chandigarh, India) and was used as the source of 1.5 kb α-amylase (*amyE*) gene. Repeated batch studies were performed in shake flasks using *E. coli* BL21(DE3). Different expression vector used in current study were pET29b, pET32b, pET43b (Invitrogen) and pCS22 [12]. Detailed strategy of cloning *amyE* gene has been reported elsewhere.

2.2. Chemicals and reagents

All the chemicals and reagents were purchased from Sigma (Sigma–Aldrich), Merck (Germany) and HiMedia (India) except when otherwise noted.

2.3. Medium and growth conditions

All cultures were grown and maintained in LB medium. *B. amyloliquefaciens* was grown at 37 °C in conical flask with shaking at 200 rpm. Repeated batch studies were carried out in shake flask and all the recombinant systems were grown without antibiotics. Initial preinoculum of all recombinant systems was prepared using their respective glycerol stocks and recombinant cultures were grown in LB medium with suitable antibiotics. Four batches were studied for each recombinant system with culture from previous batch being used as inoculum for the next batch.

2.4. Instability analysis of plasmid vector

Replica plating method was used to analyze plasmid instability by the method of Lechevalier and Corke [13]. By comparing the number of viable colonies on LB agar plate, with and without antibiotics, fraction of plasmid containing cells were analyzed and reported. All the studies in the current thesis were carried out in triplicate and experimental results represent the mean of three experimental studies.

3. Results and discussion

3.1. Plasmid loss studies in repeated batch culture

Reason for incorporating *amyE* gene into different expression vectors is to consider the metabolic load imparted on recombinant system due to gene insert and their leaky expression (data not shown) which further increases the load on the system. Reason for growing the culture for four subsequent batches was to adapt the culture to antibiotic free environment and enabling the generation of plasmid free cells. Fig. 1 shows progressive reduction in plasmid bearing cells when grown in repeated batch without any supplementation of selection pressure. Fig. 1(A–D) shows the segregational instability of plasmid with time. Plasmid pET43b-*amyE* being largest in size shows maximum instability among all vectors under study. After 42 h of studies, almost 60% of the cells were free from pET43b-*amyE* while around 35% of the recombinant cells were free from pCS22-*amyE*. This shows that plasmid size has a role to play in segregational instability by affecting the metabolic load on the host [3,14]. System with higher metabolic load tends to loose plasmid at a much faster rate.

3.2. Plasmid segregational instability

Segregational instability was interpreted using model proposed by Cooper et al. [7]. The model is based on assumptions that overall causes of plasmid instability are described by the segregational instability of plasmid R (i.e. rate at which plasmid free cells are generated from plasmid bearing cells) and the growth rate difference $d\mu$ (i.e. the difference between plasmid free and plasmid bearing cells).

Thus assuming R and $d\mu$ are constant at time t, the plasmid free proportion (p_-) of a microbial population is

$$(p_-) = \frac{(p_{-0} \cdot d\mu + R)e^{(d\mu+R)t} - R(1-p_{-0})}{(p_{-0} \cdot d\mu + R)e^{(d\mu+R)t} + d\mu(1-p_{-0})} \quad (1)$$

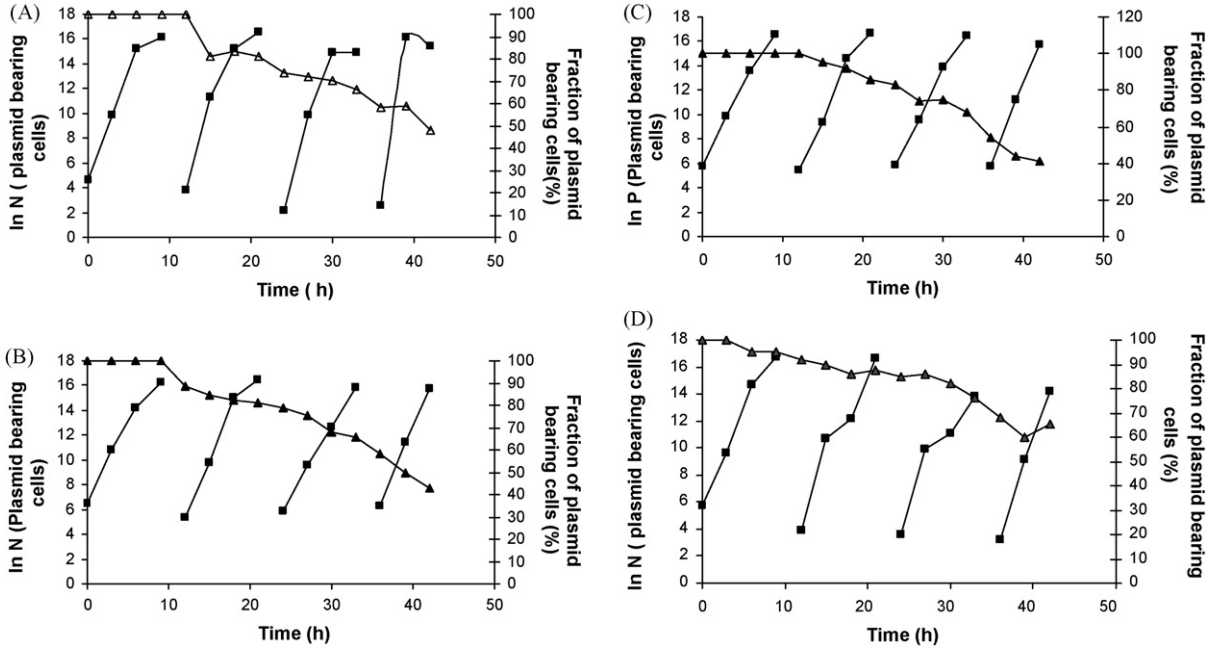

Fig. 1. Plasmid instability analyzed by replica plating in repeated batch culture for 42 h. (–▲–) Fraction of plasmid bearing cell generated during repeated batch studies; (–■–) ln N (plasmid bearing cells); (A) *E. coli*/pET29(b)-*amyE*; (B) *E. coli*/pET32(b)-*amyE;* (C) *E. coli*/pET43(b)-*amyE*; (D) *E. coli*/pCS22-*amyE*.

Table 1
Analysis of plasmid instability based on mathematical models proposed by Cooper et al. [7] and Imanaka and Aiba [8].

Recombinant system	Plasmid size (kb)	n[a]	Specific growth rate difference ($d\mu$) (gen^{-1})		α[b]	(R)[c] (gen^{-1})
			Theoretical (based on model)	Experimental		
E. coli/pET29(b)–*amyE*	6.94	79	0.021	0.00	1.00	1.9×10^{-3}
E. coli/pET32(b)–*amyE*	7.45	75	0.028	0.07	1.17	1.9×10^{-3}
E. coli/pET43(b)–*amyE*	8.76	86	0.017	0.04	1.02	2.2×10^{-3}
E. coli/pCS22–amyE	6.6	63	0.037	0.10	1.10	1.4×10^{-3}

[a] Number of generations of plasmid free cells in 42 h.
[b] The ratio of specific growth rates of plasmid free to plasmid bearing cells.
[c] Segregational instability calculated based on mathematical model.

where p_{-0}: value of p_- at time $t = 0$; t: time (in generations). Assuming $p_{-0} \ll 1$,

$$(p_-) = \frac{(p_{-0} \cdot d\mu + R)e^{(d\mu+R)t} - R}{(p_{-0} \cdot d\mu + R)e^{(d\mu+R)t} + d\mu} \quad (2)$$

Based on experimental observations, three conditions are expected

(a) $d\mu \gg R$,
(b) $d\mu \leq R$ and
(c) negative growth rate difference of magnitude greater than segregational instability ($d\mu < 0$; $|d\mu| \gg R$).

Another parameter important to mention is 'α' [8] which is the ratio of plasmid free to plasmid bearing cells. Since the experimental observation shows the value of α to be close to 1 for different recombinant systems (Table 1), it can be assumed that condition (a) from Cooper's model will be best suited for interpretation of results.

$$(p_-) \approx \left(p_{-0}\frac{R}{d\mu}\right) e^{d\mu \cdot t} - \frac{R}{d\mu} \quad (3)$$

Fig. 1(A–D) shows that $p_{-0} = 0$

$$(p_-) \approx (e^{d\mu \cdot t} - 1)\frac{R}{d\mu} \quad (4)$$

$$\ln(p_-) = d\mu \cdot t - \ln\left(\frac{d\mu}{R}\right) \quad (5)$$

Based on experimental observation, plot of $\ln(p_-)$ versus t (number of generations of plasmid free cells) was used to calculate the segregational instability of vector per generation (R) and growth rate difference ($d\mu$) of plasmid free and plasmid bearing recombinant cells.

The segregational instability shows a direct correlation with plasmid size and was found to increase with increase in plasmid size (Table 1). This can be explained by the fact that presence of plasmid increases metabolic load on host [15]. When such recombinant hosts are grown in the absence of selection pressure, plasmid free cells starts generating that compete with plasmid bearing cells for available nutrient in the medium and in subsequent generations, they outgrow recombinant cells [1,3]. This can further be confirmed with the ratio of specific growth rates of plasmid free to plasmid bearing cells (denoted as α) for all the recombinant systems under study [8]. The value of 'α' was equal to or greater than unity showing equal competence of plasmid free cells as that of plasmid bearing cells resulting in heterogeneous population. The difference in specific growth rates of plasmid free to plasmid bearing cells in heterogeneous population ($d\mu$) also confirmed the fact that specific growth rate of plasmid free cells is higher due to relatively less metabolic load as compared to plasmid bearing cells. Segregational instability was lowest for the smallest vector pCS22-*amyE* (1.4×10^{-3} gen^{-1}) while highest for pET43b-*amyE* vector (2.2×10^{-3} gen^{-1}). This shows that as the size of DNA increases,

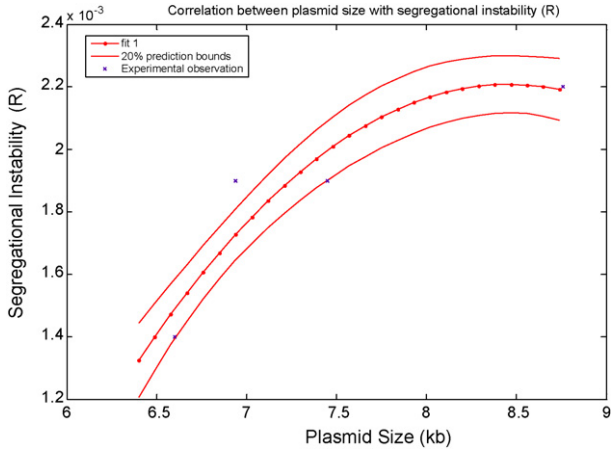

Fig. 2. Curve fitting data showing a quadratic correlation between segregational instability (R) and plasmid size (kb).

the stress on cells for maintenance of such vectors also increases, resulting in the lose plasmids when grown without any external pressure. The data is in agreement with previous reports making correlation between plasmid size and metabolic burden on the host [16].

A mathematical correlation between size of plasmid (l) and segregational instability (R) was proposed based on experimental results using MATLAB (ver. 7.4.0.287). Results show a quadratic correlation between plasmid size and segregational instability. Fig. 2 shows the curve fitting data and its correlation with calculated data. The quadratic correlation between R (segregational instability) and plasmid size (l) can be explained by the mathematical equation

$$R = -0.21 \times 10^{-3}(l)^2 + 3.5 \times 10^{-3}(l) - 13 \times 10^{-3} \quad (6)$$

The graphical correlation shows experimental observation to be closed to 20% of the predicted values. Based on the mathematical equation plasmid size at which segregational instability will be around zero is 4.67×10^{-3} kb. In the absence of any plasmid, segregational instability will be almost close to zero. In current study instability has been studied only during recombinant cell growth and not during protein expression which is reported to be a higher energy driven process than plasmid maintenance and exert higher metabolic load on cells than plasmid maintenance [17]. However for successful protein expression it is important to have a homogeneous high density culture of recombinant cell population and has been focused in current research.

4. Conclusion

This is the first report on analyzing the segregational instability of commonly used expression vectors with *amyE* gene insert. Comparison between experimental observations and mathematical interpretations of growth rate difference ($d\mu$) shows similar

trend. This is the first report that gives a new insight to correlation between plasmid size and segregational instability. A quadratic correlation was observed between size of plasmid and fraction of plasmid free cells. However, role of other cellular parameters and vector characteristics are not ruled out. Further studies are underway to evaluate the metabolic load imparted on different recombinant systems by these vectors with a focus on providing solution to vector instability using different biochemical and genetic strategies.

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