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A Comparative Study on the Recovery of *Eco*RI Endonuclease from Two Different Genetically Modified Strains of *Escherichia coli*

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A laboratory scale procedure developed for the purification of *Eco*RI restriction endonuclease was applied to two different *Escherichia coli* strains, *E. coli* 294 and *E. coli* M5248, which are genetically modified to overproduce the enzyme. The purification method consisted of three successive chromatographic steps including phosphocellulose and hydroxyapatite columns and further fractionation in a second phosphocellulose column. It was shown that the second phosphocellulose separation can be omitted in the case of *E. coli* 294. Quality control tests indicated enzyme preparations free of contaminants and endo- or exo-nucleases. The yields obtained at the final stage of the purification were 1.3x10^5 U/g cells for *E. coli* M5248 and 3.3x10^6 U/g cells for *E. coli* 294.

Key Words: *Eco*RI endonuclease, recombinant *E. coli*, purification, enzyme recovery

Introduction

Restriction enzymes have extensive applications in recombinant DNA technology. They are used in the preparation of recombinant molecules, and they provide an attractive system for the analysis of sequence specific DNA-protein interactions[1]. *Escherichia coli* RI (*Eco*RI) endonuclease is a well-known restriction enzyme that recognizes the symmetrical hexanucleotide sequence GAATTC on duplex DNA and cleaves each strand between G and A residues[2].

Physical and catalytic properties of *Eco*RI restriction endonuclease have been extensively studied by several groups and different purification protocols have been described[1−9]. In addition to the natural overproducer of *Eco*RI, *E. coli* RY13, genetically modified overproducing strains were also used to produce the enzyme. The gene encoding *Eco*RI endonuclease was placed under the control of the λp_L promoter in these genetically modified, overproducing strains[2,3,8]. The application of different purification protocols made it difficult to compare the yield and the quality of these strains in the production of *Eco*RI.

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In the present study, we compare the production of *Eco*RI endonuclease from two different genetically modified strains of *E. coli*, namely *E. coli* 294 (pPG430) and *E. coli* M5248 (pSCC2), using the same small scale purification protocols. *E. coli* 294 carries a plasmid, pPG430, in which the genes encoding *Eco*RI endonuclease and methylase are placed under the control of the lac promoter. The second strain, *E. coli* M5248, contains these genes under the control of the p_L promoter on plasmid pSCC2[2].

Materials and Methods

Bacterial Strains and Plasmids: *E. coli* 294 cells containing the plasmid pPG430, which is a derivative of pBR322, were kindly provided by Dr. Herbert Boyer (University of California, San Francisco). *E. coli* M5248 (λ bio275 cI857 HI), *E. coli* N99 (λ^+ str^+ su^-) and plasmid pSCC2 were kindly provided by Dr. Paul Modrich (Duke University, Medical Center, Durham, North Carolina).

Enzymes: T4 ligase, BamHI, PstI and PvuII used in the experimental work were purchased from New England Biolabs (USA).

Chemicals: Phosphocellulose (P11) from Whatman (UK), hydroxyapatite (HA) from Bio-Rad (USA) and acrylamide and agarose from Sigma (USA) were used in all the experiments. All other chemicals were analytical grade and supplied by either Merck AG (Germany) or Sigma (USA).

Growth of Cells: The culture medium used was LB medium containing 1% (w/v) bacto-tryptone (Difco), 0.5% (w/v) yeast extract (Difco) and 1% (w/v) NaCl. *E. coli* M5248 was cultured in LB medium supplemented with 0.01M K-phosphate (pH 7.0), 0.01% (w/v) thymine, 0.005% (w/v) thiamine and 5% (w/v) glucose. Both media were supplemented with ampicillin to a final concentration of 80 μg/ml to prevent the overgrowth of plasmid-free cells that do not have the ability to synthesise the product. Ten ml of pre-culture was used to inoculate sterile 1L LB medium and then placed in the orbital shaker at the specified temperatures for each *E. coli* strain.

The *E. coli* M5248 strain was first transformed by the plasmid pSCC2, which was obtained from the *E. coli* N99 strain, and the transformed cells were grown at 30-32°C to an absorbance value of 1.0 at 590 nm. The culture temperature was then raised to 42°C to induce product synthesis, and incubation was continued over a period of 4-5 hours as described by Cheng *et al*[2]. The cells were harvested by centrifugation at 2603 g (4000 rpm in a GSA rotor) for 15 min at 4°C and then stored at -20°C until further purification.

The second strain used in the study was *E. coli* 294, carrying the plasmid pPG430 containing *Eco*RI endonuclease and methylase under the control of the lac promoter. The *E. coli* 294 strain was grown at 37°C to an absorbance value of 1.2 at 595 nm. A parametric study was conducted to optimize induction conditions, cells were induced by the addition of 0.1mM isopropyl-β-D-thiogalactoside (IPTG), and incubation was continued over a period of 6 hours. The cells were collected by centrifugation at 2603 g for 15 minutes at 4°C. Plasmid stability of the strains was determined via the replica plating technique[10].

Purification of *Eco*RI Endonuclease:

a) Preparation of Crude Extract: *E. coli* 294 (4.375 g, wet weight) and *E. coli* M5248 (4.923 g, wet weight) were thawed, suspended in Buffer A (20mM K-phosphate which was prepared by adding 20mM KH_2PO_4 to K_2HPO_4 until the pH of the solution was neutral, 1 mM 2-mercaptoethanol, 1mM ethylenediaminetetraacetic acid (EDTA), 0.2% Triton X-100, pH 7.0) and supplemented with 0.8M NaCl and 0.1M phenyl methyl sulphonyl fluoride (PMSF) at final concentration. The cell suspensions were then sonicated while being kept on ice to prevent heating. The crude extract was dialysed for 16 hours against

Buffer A containing 0.4M NaCl, after which cell debris were removed by centrifugation at 10786 g (9500 rpm in a SS34 rotor) for 15 min at 4°C using a Sorvall RC-28S centrifuge. All steps of the purification were performed at 0-4°C.

b) First Phosphocellulose Column Chromatography: The dialysed fraction was applied to a phosphocellulose column (50cm x 2cm diameter) equilibrated with Buffer A containing 0.4M NaCl. The subsequent elution was carried out stepwise by Buffer A containing increasing concentrations of NaCl (from 0.4 to 1 M).

c) Batchwise Hydroxyapatite Chromatography: Active fractions eluted with 0.6M NaCl were pooled and applied to batchwise hydroxyapatite chromatography which was equilibrated with Buffer A containing 0.6M NaCl. The elution was carried out stepwise by increasing concentrations of K-phosphate ranging from 0.1M to 0.6M in Buffer A containing 0.6M NaCl.

d) Second Phosphocellulose Column Chromatography: The active enzyme fractions were pooled and diluted four times with Buffer A and applied to a second phosphocellulose column (10 cm x 1 cm diameter). The elution was carried out as in the first phosphocellulose column. Finally, active fractions were supplemented with 50 μg/ml BSA and dialysed against storage buffer containing 50% (v/v) glycerol, 10 μg/ml BSA, 10 mM K-phosphate, 5 mM 2-mercaptoethanol, 0.5 mM EDTA, 0.1% TritonX-100, pH 7.0.

Electrophoresis: Homogeneity of the purified enzyme was tested by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a slightly modified procedure described by Laemmli under denatured conditions[11].

Enzyme Assays: One unit of enzyme activity was defined as the amount of enzyme required to produce a complete digestion of 1.0 μg λDNA at 37°C in 1 hour in a total reaction volume of 10μl. The enzymatic activity was determined using serial dilutions of enzyme preparations. λDNA having 5 recognition sites for *Eco*RI was used as substrate in this study, and the completion of the digestion was checked on 0.8% agarose gels. The activity in units was derived from the dilution factor by determining the highest dilution that still displays complete digestion.

Protein Measurement: Protein contents of the samples were determined by the Bradford method using bovine serum albumin (BSA) as protein standard[12].

Quality Control Tests: Two different quality control tests were carried out in order to check the existence of any potential endo and exonuclease and the ligation inhibitor.

(1) Overdigestion Test: Each *Eco*RI preparation was tested for contamination by other endodeoxyribonucleases capable of digesting DNA at either random or specific sites. One microgram of substrate DNA is digested with 20 units of the enzyme for 5 hours at an appropriate temperature. This represents 100-fold excess digestion as compared to 1 unit for 1 hour as described by the manufacturers[9].

*(2) Cut-Ligate-Recut Test: Eco*RI restriction endonuclease was also tested for the presence of contaminants that would inhibit ligation or degrade termini. The restored sites were cleaved by the same enzyme. The initial cleavage of λDNA was performed with the *Eco*RI isolated in this study, and the DNA fragments were extracted by phenol and chloroform, followed by precipitation with ethanol. T4 ligase was used to ligate the fragments obtained from the initial cleavage. Ligation was performed at 16°C for 4 hours under conditions described by the manufacturer. T4 ligase was inactivated by heating the reaction mixture for 15 min at 65°C. Ligated fragments were recut by using the same enzyme preparation used in the initial cleavage of DNA[9].

Results

In the present work, *Eco*RI endonuclease was purified from both *E. coli* 294 (pPG430) and *E. coli* M5248 (pSCC2) overproducing strains using similar protocols, and the enzyme yields were compared. In plasmid pPG430, genes encoding *Eco*RI endonuclease and methylase are oriented under the control of the lac promoter[13], whereas in plasmid pSCC2, they are placed under the control of P_L^3.

a) Enzyme Purification: Table 1 summarises the purification of *Eco*RI endonuclease from *E. coli* 294 (pPG430) and *E. coli* M5248 (pSCC2). This is a modified purification method developed by Luke and Halford[6] on a genetically modified strain, *E. coli* 1100, to overproduce enzyme *Eco*RI. Luke and Halford have modified the purification used by Botterman and Zabeau[3] on the same strain, and produced a tenfold increase in the specific activity of the enzyme. Botterman and Zabeau[3] have applied the supernatant of the sonicated cell suspension to phosphocellulose and then to hydroxyapatite chromatography. The modification that Luke and Halford applied was to include a dialysis step after sonicating the cell suspension to decrease the aggregation of the protein and there fore to increase its specific activity[6]. In this work, a dialysis step was included for a similar purpose, to minimise the formation of insoluble intracellular aggregates before hydroxyapatite chromatography and also a second phophocellulose chromatography was also applied after hydroxyapatite chromatography on two different genetically modified *E. coli* strains.

Table 1. Purification of *Eco*RI endonuclease

Fractions	**Volume (ml)**	**Total Protein (mg)**	**Total Activity (U)**	**Specific Activity (U/mg)**	**Recovery (%)**	**Purification Fold**
***E. coli* 294 strain (4.375 g wet cells)**						
Supernatant of disintegrated cell suspension	100	356.875	$1.5x10^7$	42 031	100	1.0
First phosphocellulose chromatography	84	43.75	$1.47x10^7$	336 000	98	8.0
Batchwise hydroxyapatite chromatography	48	13.775	$1.44x10^7$	1 045 372	96	24.9
Second phosphocellulose chromatography	55	9.023	$9.6x10^6$	1 066 718	64	25.4
***E. coli* M5248 strain (4.923 g wet cells)**						
Supernatant of disintegrated cell suspension	150	932	$1.05x10^6$	1 127	100	1.0
First phosphocellulose chromatography	80	64	$8.9x10^5$	13 906	85	12.3
Batchwise hydroxyapatite chromatography	48	24	$7.5x10^5$	30 992	71	27.5
Second phosphocellulose chromatography	64	6.1	$6.4x10^5$	104 918	60	93.1

Cells that were induced for the synthesis of *Eco*RI either by the addition of 0.1 mM IPTG in the case of *E. coli* 294 (pPG430) or by a temperature shift at 42°C for a period of 5 hours in the case of *E. coli* M5248 (pSCC2), were disrupted by sonication. The crude extracts were dialysed, and cell debris was removed together with precipitated proteins by centrifugation. The supernatant was applied to a phosphocellulose column as described in the Materials and Methods section. The elution profiles for *E. coli* 294 and *E. coli* M5248 are given in **Figures 1 and 2**, respectively. The active fractions were pooled and applied to batchwise HA chromatography, which resulted in 71 and 96% recovery of *Eco*RI endonuclease with *E. coli* M5248 and *E. coli* 294 respectively (**Figure 3**). The application of a second phosphocellulose column only increased the specific activity of *Eco*RI threefold in the case of *E. coli* M5248 by eliminating contaminating proteins (**Figure 4**). Since the degree of purification was not improved any further by a second phosphocellulose column in the case of *E. coli* 294, it was concluded that the application of the second phosphocellulose column could be omitted. This observation allowed the development of a simple two-step procedure consisting of only two chromatographic separations for the preparation of pure *Eco*RI for commercial use from the genetically engineered overproducing *E. coli* 294.

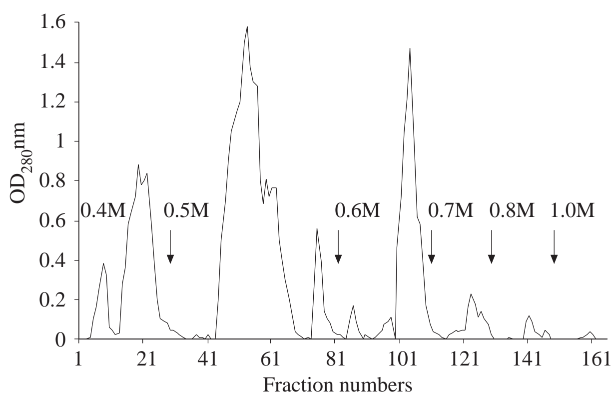

Figure 1. Elution profile of the first phosphocellulose column chromatography for the *E. coli* 294 strain. Arrows indicate the points where the buffer has been changed.

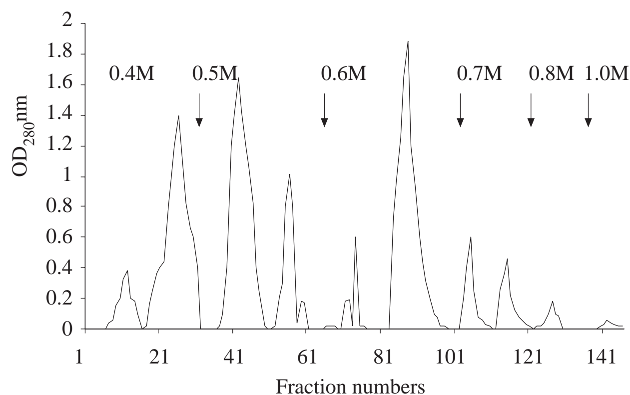

Figure 2. Elution profile of the first phosphocellulose column chromatography for the *E. coli* M5248 strain. Arrows indicate the points where the buffer has been changed.

The activity measured in the clarified cell extract of the *Eco*RI enzyme isolated from *E. coli* 294 (pPG430) was found to be 3.43x10^6 U/(g-wet cells) whereas it was 2.13x10^5 U/(g-wet cells) in the case of *E. coli* M5248 (pSCC2). The existence of a 16-fold difference in the clarified cell extracts has clearly indicated that *E. coli* 294 with pPG430 is a better source for the efficient production of *Eco*RI endonuclease. The application of a two-step protocol for *E. coli* 294 (pPG430) and a three-step protocol for *E. coli* M5248 (pSCC2) resulted in enzyme yields of 3.3x10^6 units and 1.3x10^5 units of *Eco*RI endonuclease per gram wet cells with 96% and 61% recovery respectively. 3.1 and 1.2 mg of final product were obtained per gram of wet cells with specific activities of 1x10^6 U/mg and 1x10^5 U/mg from *E. coli* 294 (pPG430) and *E. coli* M5248 (pSCC2), respectively. SDS-PAGE analyses of the enzyme preparations obtained from the two different overproducing strains showed patterns identical to that of commercial *Eco*RI (**Figure 5**).

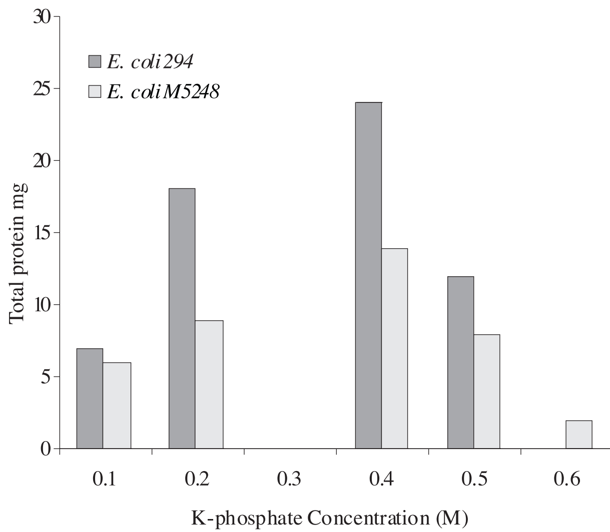

Figure 3. The change in total protein concentraiton with respect to K-phosphate concentration in hydroxapatite chromatography

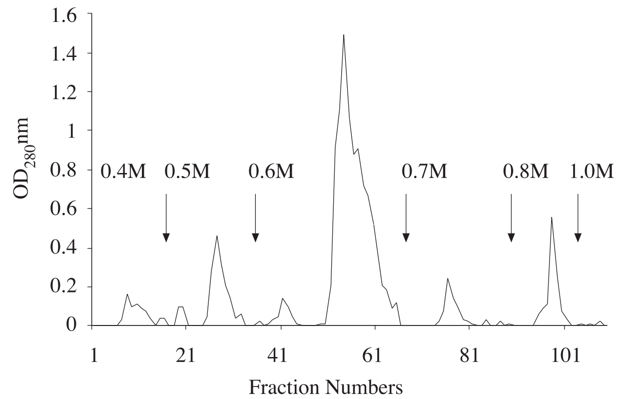

Figure 4. Elution profile of the second phosphocellulose column chromatography for the *E. coli* M5248 strain. Arrows indicate the points where the buffer has been changed.

b) Quality Test Results: An overdigestion quality test indicated the absence of endo- and exonucleases in the final enzyme preparations (**Figure 6**). The same *Eco*RI preparations were also tested for their ability to ligate, and recut restriction fragments of λDNA and were found to be free of contaminants that would inhibit ligation or degrade termini (**Figure 7**). These results have clearly shown that the enzyme preparations were suitable for use in molecular biology.

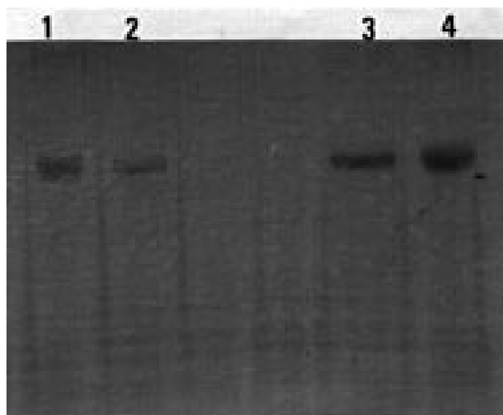

Figure 5. SDS-PAGE analysis of the purified *Eco*RI endonuclease

Lane 1 and 4: Commercial *Eco*RI endonuclease

Lane 2: *Eco*RI endonuclease purified from the *E. coli* M5248 (pSCC2) strain

Lane 3: *Eco*RI endonuclease purified from the *E. coli* 294 (pPG430) strain

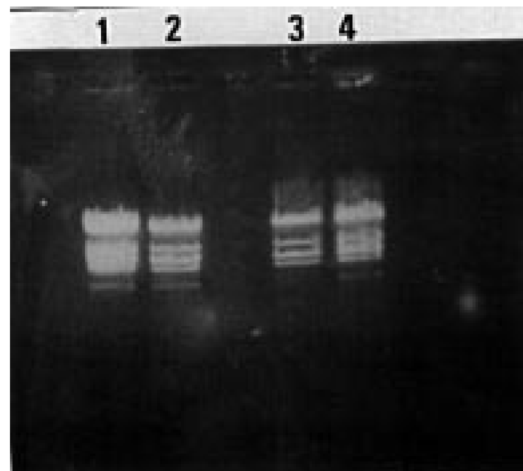

Figure 6. Overdigestion of λDNA by *Eco*RI endonuclease purified from the *E. coli* 294 (pPG430) (lanes 1 and 2) and the *E. coli* M5248 (pSCC2) strain (lanes 3 and 4)

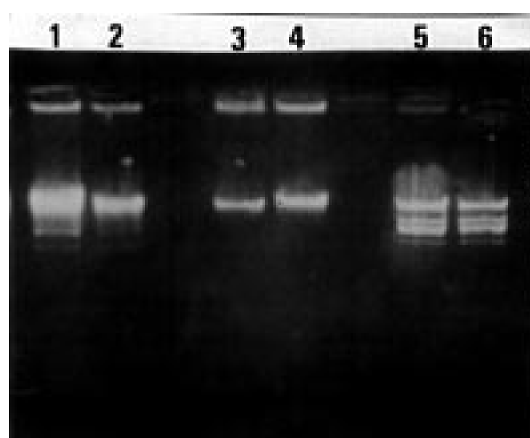

Figure 7. Ligation-Recut analysis of *Eco*RI endonuclease
Lane 1: Initial cleavege of λDNA by *Eco*RI endonuclease purified from the *E. coli* M5248 (pSCC2) strain
Lane 2: Initial cleavege of λDNA by *Eco*RI endonuclease purified from the *E. coli* 294 (pPG430) strain
Lane 3: Ligation of DNA fragments produced by *Eco*RI endonuclease purified from the *E. coli* M5248 (pSCC2) strain
Lane 4: Ligation of DNA fragments produced by *Eco*RI endonuclease purified from the *E. coli* 294 (pPG430) strain
Lane 5: Recut of ligated fragments by *Eco*RI endonuclease purified from the *E. coli* M5248 (pSCC2) strain
Lane 6: Recut of ligated fragments by *Eco*RI endonuclease purified from the *E. coli* 294 (pPG430) strain

Discussion

The restriction endonuclease *Eco*RI has been purified in many laboratories using different purification procedures. Greene *et al.*[5] have developed a method for the purification of *Eco*RI endonuclease from *E. coli* RY 13 strain. Their yield was 13 U/gcell. Modrich*et al.*[1] have modified this method to increase the yield of the enzyme to 190 U/gcell from the same strain. Vlaktais and Bouritis[14] have purified *Eco*RI endonuclease from the same strain by applying sequence specific DNA affinity chromatography and ended up with a yield of 1.8x10^5 U/g cell. Mehra *et al.*[9], on the other hand, have applied the dye-ligand chromatography to purify *Eco*RI endonuclease from *E. coli* RY 13. Their yield was 3x10^4 U/g cell. Cheng *et al.*[2] have constructed an overproducing strain, *E. coli* M5248 (pSCC2), to purify *Eco*RI restriction and modification enzymes. Their purification method involves streptomycin and ammonium sulphate fractionations followed by phosphocellulose and hydroxyapatite chromatographies respectively. They have obtained 500 mg of enzyme per kg cell paste with a recovery of 47%. The specific activity of the *Eco*RI endonuclease was reported to be 4.5x10^4 U/mg protein. It can be calculated that Cheng *et al.*[2] have obtained approximately 2.25x10^4 units of enzyme per gram cell. In the present study, an almost tenfold increase was obtained in the yield of the *Eco*RI endonuclease from *E. coli* M5248 (pSCC2) by the application of a new purification scheme.

On the other hand, the yield and the specific activity of the enzyme produced by *E. coli* M5248 (pSCC2) in this work was at a lower level when compared with the results reported by Luke and Halford[6]. These investigators have used a different overproducing construct in which the gene encoding *Eco*RI was placed under the control of the same p_L promoter, but the genes carrying the *Eco*RI methylase and cI-coded temperature sensitive repressor were on separate compatible plasmids. There are substantial differences in

both enzyme yield and enzyme specific activity between the two strains used by Cheng *et al.*[2] and Luke and Halford[6], and these may be due to the lower expression of the M5248 (pSCC2) strain, resulting either from the distance between the p_L promoter and the gene for *Eco*RI endonuclease or from the simultaneous placement of *Eco*RI and methylase genes on the same plasmid. The nature of the host cells and the plasmid copy number may be other important factors that lead to lower enzyme recovery in the case of the *E. coli* M5248 (pSCC2) strain.

It has to be noted that several improvements may be possible at the fermentation level to improve the productivities of *Eco*RI endonuclease. A careful investigation of growth characteristics, recombinant gene expression and plasmid stability in these recombinant strains may allow the development of good model systems for predicting the yield of recombinant protein production in induced cultures.

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References

1. P. Modrich and D. Zabel, *Eco*RI Endonuclease: Physical and Catalytic Properties of the Homogeneous Enzyme, **J. Biol. Chem.**, **251**, 5866-5874 (1976).
2. S. Cheng, R. Kim, K. King, S. Kim, P. Modrich, Isolation of Gram Quantities of *Eco*RI Restriction and Modification Enzymes from an Overproducing Strain **J. Biol. Chem.**, **259**, 11571-11575 (1984).
3. J.H. Botterman and M. Zabeau, High Level Production of the *Eco*RI Endonuclease Under the control of the pL promoter of the Bacteriophage Lambda, **Gene**, **37**, 229-239 (1985).
4. R.A. Rubin and P. Modrich, Purification and Properties of *Eco*RI Endonuclease, **Methods in Enzymology**, **65**, 96-110 (1980).
5. P.J. Greene, H.L. Heyneker, F. Bolivar, R.L. Rodriguez, M.C. Betlach, A. Covarrubias, lK. Backman, D.J. Russel, R. Tait, H.W. Boyer, A general method for the purification of Restriction Enzymes, **Nucl. Ac. Res.**,**5(7)**, 2371-2380 (1978).
6. P. Luke and S. Halford, Solubility of the *Eco*RI Restriction Endonuclease and its Purification from an Overproducing strain, **Gene**, **37**, 241-246 (1985).
7. A. Bingham, A.F. Sharman, T. Atkinson, The Purification of Restriction Endonuclease *Eco*RI by Precipitation Involving Polyethyleneimin, **FEBS Letters**, **76(9)**, 250-256 (1977).
8. J.H. Botterman, D.R. De Buyser, J.A. Spiert, M. Zabeau, G.C. Vansteenkiste, Fermentation of Recovery of the *Eco*RI Restriction Enzyme with a Genetically Modified *Escherichia coli* Strain, **Biotechnol. Bioeng.**, **27**, 1320-1327 (1985).
9. R.S. Mehra, V.P. Malhatra, G.W. Rembhatkar, Rapid Purification of a Restriction Endonuclease from *Escherichia coli* RY13, **Biotechnology Techniques**, **7(6)**, 411-414 (1993).
10. M.M. Ataai and M.L. Shuler, A mathematical model for Prediction of plasid copy number and Genetic Stability in *Escherichia coli,* **Biotechnol. & Bioeng.**,**30**, 389-397 (1989).
11. U.K. Laemmli, Cleavage of Structural Proteins During the Assembly of the T4, **Nature**, **227**, 680-685 (1970).

12. M. Bradford, A rapid and Sensitive Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding, **Anal. Biochem.**, **72**, 248-254 (1976).

13. C. Tamerler, Yıldır, Z. İlsen Önsan, B. Kırdar, Optimization of starting time and period of induction and inducer concentration in the production of *Eco*RI Restriction Enzymes from E.coli 294, Strain, **Turk. J. Chem., 22,** 221-226 (1998).

14. G. Vlatakis and V. Bouritos, Sequence-specific DNA Affinity Chromatography: Application to the Purification of *Eco*RI and **Sph**I, Methods in Enzymology, 352-358 (1991).