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Extraction of Dengue 2 Plasmid DNA Vaccine (pD2) from Cell Lysates by Aqueous Two-Phase Systems

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Abstract: This research describes the partitioning in PEG/phosphate systems of the plasmid pD2, a dengue 2 plasmid DNA vaccine, present in a clarified *E. coli* alkaline lysate. Factors that affect the partition as PEG molecular weight, plasmid concentration and the lysate volume loaded in the system were investigated. Results showed that partition behavior of plasmid DNA depends on the system molecular weight, a considerable amount of protein of the cell lysate was accumulated in the interphase of the systems. The best recovery plasmid yield (37%) was obtained with PEG 400 (20/20% w/w) systems with a 60% (w/w) lysate load.

Key words: Aqueous two-phase systems, liquid-liquid extraction, plasmid DNA

INTRODUCTION

The widespread interest in gene therapy and DNA-based vaccination has led to an increased demand for large amount of pure plasmid DNA (Ribeiro *et al*., 2000; Mountain, 2000; Wang *et al*., 2001). It is urgent to develop new methods to purify plasmids with high yields and minimal or no contamination (Trindade *et al*., 2005). The cost of the recovery of products with importance for pharmaceutical/clinical research for industrial use becomes critical to the overall process economics, representing 50-90% of the total cost (Prazeres *et al*., 2001; Moreira *et al*., 2005). The purification of plasmid DNA is thus usually accomplished by a sequence of three to four different purification steps depending on the level of purity required (Diogo *et al*., 2000; Lemmens *et al*., 2003). Aqueous Two-Phase Systems (ATPS) had been widely and successfully used on the extraction and purification of biological macromolecules (Rito-Palomares, 2004). Over 50 years ago, Albertsson (1962) was one of the first to study the separation of biomolecules and particles in ATPS in a systematic way. The technique later proved to be of immense utility in analytical, biochemical and environmental research and applications (Rito-Palomares and Middelberg, 2002). As opposed to proteins, plasmid purification by aqueous two-phase extraction has evolved very little and few related have appeared in the literature in the last years (Ribeiro *et al*., 2002; Kepka *et al*., 2004; Trindade *et al*., 2005). Aqueous two-phase systems are formed by mixing two polymers or a polymer and salt above some threshold concentration. Both phases contain a high proportion of water (80-95%) providing a nontoxic environment for biomolecules and low interfacial tension, they provide mild conditions especially suited for biological macromolecules separation (Shang *et al*., 2004; Rito-Palomares, 2004). Separation is achieved by the different distribution, between the two phases, of the target compound and the contaminants. The mechanism of partition is not well understood and separation of compounds is usually attained by a systematic variation of system composition (Hatti-Kaul, 1999; Shang *et al*., 2004). This includes type, molecular weight and concentration of polymer, type and concentration of salt and pH. The partition of nucleic acids in ATPS depends on many factors, such as the size and chemical properties

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of the macromolecule, the properties of the system components and the ionic composition (Kepka *et al*., 2004). A change in the systems properties will change the surface properties of the partitioning solutes and thus affects partitioning. Some of the interactions between solutes and phase components must involve hydrogen bonds, charge interaction, van der Waals forces, hydrophobic interaction and steric effects (Albertsson, 1986). Overall, to predict the partitioning is a difficult task, particularly in the case of large molecules (Ribeiro *et al*., 2002). Here we describe the partitioning of a plasmid pD2, a dengue 2 plasmid DNA vaccine, present in a clarified *E. coli* alkaline lysate, in PEG/phosphate systems. Factors that affect the partition, PEG molecular weight, plasmid concentration and the lysate volume loaded the system were investigated.

MATERIALS AND METHODS

Polyethylene glycol (PEG) 300, 400, 550, 1000 and 8000 were purchased from Sigma Chemical Company (St Louis, MO, USA). Di-potassium hydrogen phosphate was from Nuclear (São Paulo, Brazil). Pico Green® ds DNA quantization reagent was acquired from Molecular Probes (Leiden, The Netherlands). In all experiments were applied the Plasmid pD2 a dengue 2 plasmid DNA vaccine, expressing the virus pre-membrane and envelope proteins (Lu *et al*., 2003). All the other reagents used were of analytical grade.

Plasmid and bacterial strain: The plasmid was transformed and propagated in *Escherichia coli* XL1 Blue. Recombinant bacteria were stored in 25% (v/v) glycerol at -80°C (Sambrook *et al*., 1989).

Production of bacterial culture: Bacteria were grown overnight in 1000 mL shake flasks containing 250 mL of Terrific Broth medium (20 g tryptone L^{-1}, 24 g yeast extract L^{-1}, 4 mL glycerol L^{-1}, 0.017 M KH_2PO_4, 0.072 M K_2HPO_4) supplemented with 30 μg mL^{-1} of kanamycin, at 37°C and 160 rpm. *E. coli* XL1 Blue cells without plasmid were grown in similar conditions but without antibiotic.

Cell lysis: A modified alkaline method was used for cell lysis (Sambrook *et al*., 1989). Cells (250 mL) were harvested by centrifugation at 15,000 g (20 min, 4°C) the pellets were resuspended in 12.5 mL of 50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0. The cells were lysed by adding gently (10 min on ice) 12.5 mL of 200 mM NaOH 1% (w/v) SDS during 10 min on ice. The lysate was neutralized with 9.4 mL of a solution of 3 M potassium acetate, 11.5% (v/v) glacial acetic acid (10 min on ice). All the solutions were previously chilled. The precipitate was removed by centrifugation (15,000 g, 30 min, 4°C) and the lysate was kept at -20°C for further plasmid DNA recovery and purification with ATPS.

Plasmid standards: Plasmid standards were prepared from *E. coli* cultures using the Flexi Prep kit, GE Healthcare, resuspended in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and quantified by measuring the absorbance at 260 nm.

Aqueous two-phase systems: The PEG/K_2HPO_4 ATPS were prepared with typical concentrations for the chosen PEG molecular weights: PEG 300 (20/20% w/w), 400 (20/20% w/w), 550 (20/20% w/w), 1000 (15/13% w/w) and 8000 (10/10% w/w). The systems (5 g total mass) were prepared in conical 15 mL centrifuge tubes by adding of clarified cell lysate with plasmid (pD2), loaded was 1, 2 or 3 g (corresponding to 20, 40 or 60% w/w to the ATPS). After the addition of all the components of the systems was vortex mixing. The systems were centrifuged for 1 min at 1000 g to facilitate phase separation. Top and bottom phases were carefully isolated and stored at 4°C for further analysis. Each phase (15 μL) was analyzed by electrophoresis in 1% agarose gels run with TAE buffer in the presence of 0.5 μg mL^{-1} ethidium bromide. The controls of the aqueous two phase systems of phases had been made with the same composition of the ones described above using the *E. coli* lysate without plasmid (XL1 Blue lysate). The procedures followed to obtain top and bottom phases were the same.

Plasmid DNA analyses: Plasmid DNA was quantified by fluorescence analysis using Pico Green® (Molecular Probes, Inc., USA) an ultra-sensitive fluorescent stain that binds specifically to double-stranded nucleic acids. Before each set of measurements, a Pico Green® stock solution as per manufacture's instructions was prepared. The fluorescence was measured in 490 nm excitation and 520 nm emission, using a spectrometer (Yvon Jobin, France) connected to a laser (Innova/Coherent, USA). In order to quantify the plasmid in the pD2 lysate, a calibration curve was made using the lysate without plasmid (XL1 Blue lysate). The XL1 Blue lysate was diluted using sterile TE buffer. Calibration standards (5-60 ng mL^{-1}) were prepared by adding known amounts of pure pD2 plasmid to this diluted XL1 Blue lysate. For the quantitatation of plasmid in the ATPS blanks, the blanks top and bottom phases were diluted with sterile TE buffer. Calibration standards (5-60 ng mL^{-1}) were prepared by adding known amounts of pure pD2 plasmid to these diluted top and bottom phases.

Protein measurements: The top and bottom phases of the system were analyzed for protein content with the Bicinchoninic Acid (BCA) Protein Assay according to the manufacturer's instructions (Pierce, Rockford, IL, USA). To overcome the interference of PEG and salt in the samples, a series of calibration curves were constructed with appropriate ATPS blanks prepared as follows. A mixture of the buffers used in the preparation of the lysates (here after named mixture X) with exactly the same final composition was made. The blanks were then prepared by replacing the lysate in the ATPS preparation with the mixture X. Top and bottom phases were carefully separated and kept at 4°C. Calibration curves were then carried out by adding bovine serum albumin (concentrations up to 250 μg mL^{-1}) to each top and bottom phases of the previously prepared ATPS blanks. The calibration curve used for the quantization of protein in the lysate was made by adding BSA directly to the mixture X. For analysis, 100 μL of each sample were mixed with 100 μL of sodium deoxycholate (0.15% w/v) with 800 μL sterile distilled water. After 10 min at room temperature, 100 μL of trichloroacetic acid (72% w/v) was added. Samples were then vortex mixed and centrifuged for 20 min at 8,000 g. The supernatant was removed and pellets solubilized in 50 μL of sodium dodecyl sulphate (5% w/v) containing 0.1M NaOH, then BCA reagent was added (200 μL) and the samples were incubated at 60°C for 30 min. Absorbance was measured at 595 nm in Bio-Rad (Hercules, CA) model 550 micro plate reader.

RESULTS AND DISCUSSION

Plasmid and RNA partitioning: In systems of aqueous two-phase composed by PEG 300, 400 and 550 for concentrations of 20 and 40% (w/w) of lysate a well-defined white interphase was observed (Table 1). For 60% (w/w) concentration a white interphase was also observed in systems with PEG 300, 400, 550 and 1000. Agarose gel analysis of the interphase material (data not shown) confirmed that, in all systems, plasmid and RNA were lost to in the interphase area. Studies carried out by Kimura (2000) with potassium phosphate-PEG aqueous two-phase system (PEG 1500 and 3000) in the RNA partition, showed that the RNA of low-molecular-mass was partitioned between the top and bottom phases, if partitioned alone. However, the RNA low-molecular-mass was caught in the interphase to a significant extent, if partitioned with the coexisting RNA high-molecular-mass. In the current study, the studied systems, the RNA was partitioned towards the systems where the plasmid was partitioned (Fig. 1A, B and C). Probably the RNA that constitutes cell lysate has low-molecular-mass, therefore partitioned between the two phases (top and bottom), depending on the PEG molecular weight of the system. By the analysis in the gel of agarose it was observed that the plasmid was partitioned in the PEG rich higher phase for PEG 300 and 400, while for PEG 1000 and PEG 8000 systems the plasmid was partitioned in the salt rich phase (Fig. 1 A, B and C). However, using PEG 400 (60% w/w) systems probably the plasmid was to the interphase area. In these figures the plasmid shows two conformations in the cell lysate solution in the ATPS, one corresponding to the plasmid in its open circular (oc) and the other in a supercoilded (sc) form. Probably these forms are due to the proper cellular process of lyse, by the presence of high concentrations of salt and others ion presents in the solution or as result of the plasmid instability (Ribeiro *et al*., 2000). The partitioning of plasmid in ATPS is complex and influenced by a large number of factors (Ribeiro *et al*., 2002). In PEG-salt systems, one of the major factors is the interaction that exists between the components (other than the solute) in each phase. In these systems the energy of each phase that arises from these interactions is considerably different. Top phases are dominated by the repulsive interaction between PEG and salt and bottom phases by the strong attraction of salt to water. Accordingly, solutes (plasmid and RNA molecules, in this study) will prefer the top phase that has PEG with low molecular weight, since disrupting interactions between its components is energetically favorable (Trindade *et al*., 2005). The type, salts concentration and the ratio between different ions in both

Table 1: Concentrations of plasmid and protein in the lysate and after extraction with ATPS

System	Lysate load (%) (w/w)	Plasmid (μg mL^{-1})	Recovery plasmid yield (%)	Protein (μg mL^{-1})	Recovery protein yield (%)
Lysate	-	238.0±2.8	100.00	986.0±4.6	100.00
PEG 300	20	52.0±1.4	21.8±0.7	78.9±4.7	8.0±1.4
Top phase	40	53.0±3.2	22.2±0.6	411.1±5.0	41.7±0.5
	60	67.0±1.4	28.1±0.7	354.9±4.2	36.0±1.4
PEG 400	20	82.0±2.1	34.4±0.5	177.4±1.8	18.0±0.7
Top phase	40	83.0±2.1	34.9±0.8	364.8±3.6	37.0±0.7
	60	88.5±1.0	37.1±0.4	640.9±2.9	65.0±1.1
PEG 1000	20	25.0±1.4	10.5±0.2	39.4±1.1	4.0±0.1
Bottom phase	40	26.0±2.1	10.9±0.2	78.9±0.8	8.0±0.2
	60	32.0±1.4	13.4±0.8	78.9±1.5	8.0±0.2

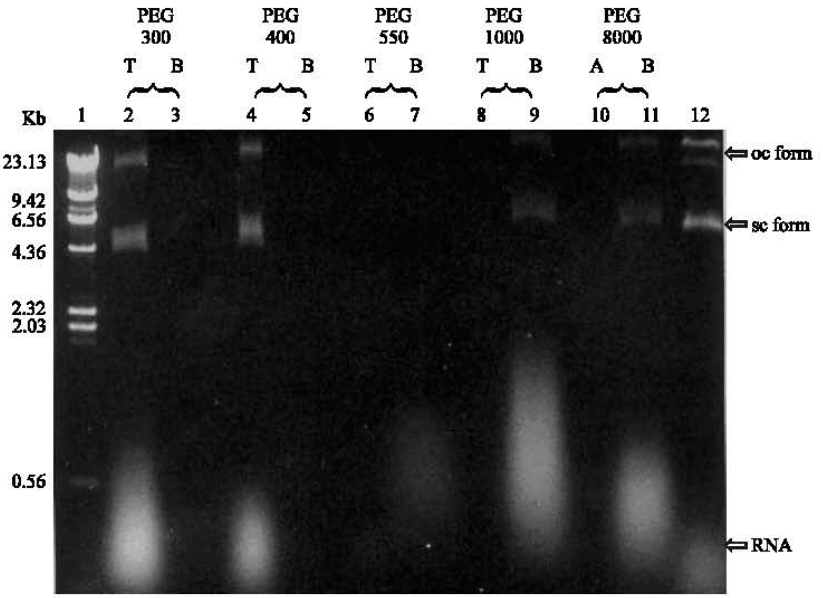

Fig. 1A: Agarose gel analysis of plasmid and RNA partitioning in ATPS with 20% (w/w) of lysate. T-top phase. B-bottom phase. Lane 1.1 μg mL^{-1} of Lambda DNA/*Hind* III marker, lanes 2 to 11:15 μL of each of the indicated phase, lane 12:15 μL of the lysate. OC: open circular. SC: supercoiled

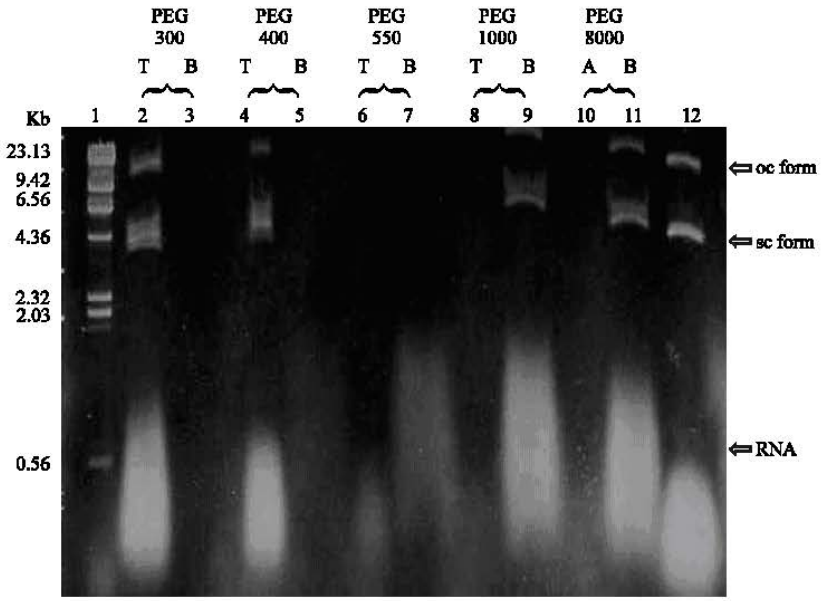

Fig. 1B: Agarose gel analysis of plasmid and RNA partitioning in ATPS with 40% (w/w) of lysate. T-top phase. B-bottom phase. Lane 1.1 μg mL^{-1} of Lambda DNA/*Hind* III marker, lanes 2 to 11:15 μL of each of the indicated phase, lane 12:15 μL of the lysate

phases, is particularly important for highly charged molecules such as nucleic acids (Albertsson, 1986). The partition of a charged solute is influenced by the unequal distribution of ions due to different affinities for the phases, which generates an electrical potential between the phases, $\Delta\psi$, defined as ψ_{top}-ψ_{bottom}. The magnitude and sign of $\Delta\psi$ are determined by the partitioning behavior of ions from the majority abundant salt in the system. In PEG-phosphate systems, the phase-forming salt will, therefore, determine $\Delta\psi$. In these systems $\Delta\psi$ is positive, for the PEG-phosphate and PEG-sulphate systems, so that it favors partitioning of net negatively

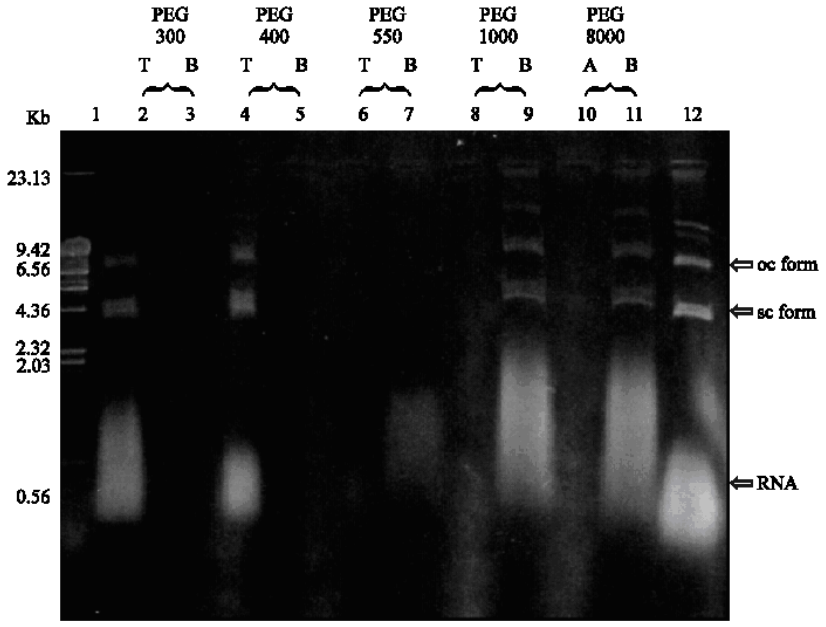

Fig. 1C: Agarose gel analysis of plasmid and RNA partitioning in ATPS with 60% (w/w) of lysate. T-top phase. B-bottom phase. Lane 1.1 μg mL^{-1} of Lambda DNA/*Hind* III marker, lanes 2 to 11:15 μL of each of the indicated phase, lane 12:15 μL of the lysate

charged biomolecules into the PEG-rich top phase (Johansson *et al*., 1998). The phenomena of the collapse of DNA macromolecules in aqueous solutions of PEG can also play a role in the partitioning of plasmid to the salt-rich phase. At low PEG molecular weight (or low PEG concentrations) the flexible polymer chains can penetrate inside of the DNA which adopts a swollen coil conformation and regime of good compatibility between PEG and DNA. If the PEG molecular weight is higher (or if more PEG is added) the solvent quality for DNA becomes poorer and the effective attraction between DNA segments in the macrolecules increases. At a certain point, a discrete transition occurs when the DNA supercoiled contracts abruptly to form a compact globular structure. In this regime of perfect incompatibility, there is segregation between DNA chains and PEG molecules (Vasilevskaya *et al*., 1995).

Protein partitioning: In systems with 20% (w/w) of lysate (Table 2) it was observed a great accumulation around 90-76% of protein (PEG 300 to 1000) in the interphase. While for systems with PEG 8000 the partitioned protein was around 60%. It was also observed that in all the studied systems and all for the concentrations of cell lysate occur partition of the protein for both the phases, top and bottom, except in PEG 300 system (20% (w/w) of cell lysate) it did not occur partition for the low phase. With the increase of the loaded volume of cell lysate occurs reduction of the accumulation of protein in the interphase. These results are in accordance with published studied, which revealed that the majority of intracellular proteins show changes in preference phase when it was partitioned in PEG-phosphate systems with PEG molecular weight between 1000 and 2000 (Albertsson, 1962). The preference for the top phase seen for pH values above the proteins isoeletric point is lost when higher PEG molecular weights are used due to excluded volume effects (Huddleston *et al*., 1990). Shibusawa *et al*. (2003) using aqueous two-phase systems PEG 1000 (16%)/potassium phosphate (12.5%) carry on purification of single-strand DNA binding protein from an *Escherichia coli* lysate demonstrated that purification occurs using this solvent system, which has conventionally been used to separate several proteins. We select for this study systems formed by PEG 300, 400 and 1000 the lives promising ones will be plasmid isolation and therefore, will be selected to further partitioning studies. The systems PEG 300, 400 and 1000 were selected for the owed partition of proteins the characteristics of these systems in partition the protein for the interphase, so that a future application of these systems as pre-purification method or even of purification, these systems will be selected due to selective partition of the plasmid of the protein contaminants originating from of the lysate.

Table 2: Protein partitioning in PEG phosphate ATPS lysate

		Partition protein (%)				
Lysate (%)	System	300	400	550	1000	8000
	Top phase	8.0±0.7	18.0±1.8	19.0±0.8	20.4±0.6	12.5±0.4
20	Interphase	92.0±0.2	80.5±0.6	78.0±0.7	75.8±0.5	59.0±1.4
	Bottom phase	0.5±0.0	1.5±0.3	3.0±0.3	3.8±0.4	28.5±1.0
	Top phase	41.7±0.9	37.0±1.2	58.0±0.4	34.3±0.6	8.8±0.3
40	Interphase	56.1±0.6	56.0±1.4	37.0±0.5	57.7±1.0	45.9±0.6
	Bottom phase	2.2±0.3	7.0±0.2	5.0±0.2	8.0±0.0	45.3±0.4
	Top phase 3	34.0±1.1	65.0±1.1	72.4±1.7	57.6±1.3	20.0±0.9
60	Interphase	60.4±1.1	33.0±1.2	22.0±0.7	34.2±0.6	33.0±0.7
	Bottom phase	5.6±0.4	2.0±0.1	5.6±0.2	8.2±0.4	47.0±1.4

Effect of lysate load: The effect of lysate load (20, 40 and 60% of total system mass) on plasmid extraction yield was analyzed for each of the selected ATPS. The plasmid in the cell lysate and in the top and bottom phases obtained after extraction was quantified by fluorescence analysis. Calibration curves were constructed for all the systems tested. The slopes were obtained from the corresponding linear regressions. The interception values constitute an indication of the amount of impurities (gDNA, RNA and proteins) present in the phase, which increase the fluorescence signal. In all systems this value increased with an increase in the lysate load. These observations are in accordance with the agarose gel analysis (Fig 1A, B and C) that shows a significant increase in RNA concentration with the lysate load for the bottom phase of systems PEG 1000. Ribeiro *et al*. (2002) working with plasmid pCF1-CFTR isolated in PEG/salts phosphate systems obtained similar results to the presented ones in the present work. The amount of lysate loaded to the systems (20, 40 or 60%) also affects the partitioning (Table 1). In Table 1 the results of the recovery plasmid and the protein are presented. It is observed that with increased of the loaded volume in the systems it is increased recovery of the plasmid and the protein. The loss of plasmid to the interphase is responsible for the low recovery yields obtained in some of the systems. The addition of increasing volumes of cell debris and components, changes the position of the binodal curve in phase diagrams by displacing it towards the origin (Hatti-Kaul *et al*., 1999; Huddleston *et al*., 1991). This means that for each PEG molecular weight, the difference in composition of the top and bottom phases increases with the lysate load, providing an increased driving force for the unequal partition of the solute (Johansson *et al*., 1998). This was observed for plasmid partitioning with all the used systems where the plasmid recovery yield increased with the lysate load.

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