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Original Article

Validation of a Simple and Rapid Method for Assessment of Intracellular Bacterial Asparaginase

Mahmood Barati [a,b*], Maryam Tabarzad [c], Hossein Safarpour [d], Ali Ghanbariasad [e], Omar Ghaderi [f]

[a] *Department of Pharmaceutical Biotechnology, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran*

[b] *Student research committee, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran*

[c] *Protein Technology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran*

[d] *Pharmaceutical Sciences Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran*

[e] *Department of Medical Biotechnology, School of Medicine, Fasa University of Medical Sciences, Fasa, Iran*

[f] *Department of Pharmaceutical Biotechnology, School of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran*

Abstract

L-Asparaginase has remarkable properties which make it useful in dual pharmaceutical and food industries.In this study, simple and advantageous methods have been validated for rapid and precise determination of intracellular L-Asparaginasein bacterial species. A suspension of bacterial cells was used instead of cell extract and incubated by substrate (asparagine) after simple wash and centrifugation steps. Due to loss of enzyme activity which could be caused by cell distruption methods such as sonication or enzymatic treatment, cell suspension was used instead of the cell extract. Thus, this method not only is cost effective but also speeds up the screening process and leasd to higher measurement accuracy. To validate this method, two species of bacteria; *E.coli* ATCC 8739 and *Halomonas H28* were used. After cultivation, the cells were harvested and washed. Then, 5 serial dilutions were prepared from each bacterium, and the asparaginase activity in each of them was measured by methods including sonication, enzymatic lyses, and the cell suspension. The results have showed that the changes in asparaginase activity in all 5 serial dilutions are linear and there is good agreement between the sonication and the cell suspension methods. Also, it was shown that activities measured by the enzymatic method were significantly higher than the other two methods.

Keyword: Enzymatic lyses, Intracellular, L-Asparaginase, Sonication, *E. Coli*, *Halomonas*.

Corresponding Author: Barati M, Department of Pharmaceutical Biotechnology, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Student research committee, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
Tel: (+98)21-22426508
E-Mail: mahmood.barati@gmail.com
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1. Introduction

L-Asparaginase (E.C.3.5.1.1) catalyzes the conversion of L-asparagine to L-aspartate and ammonia. It has remarkable properties which make it useful in dual pharmaceutical and food industries. In the medicine field, it has been used as a main treatment of acute lymphoblastic leukemia (ALL) [1-3] and in food industry as an agent to prevent the formation of acrylamide (a potential neurotoxin and carcinogenic agent) in fried and heated foods [4-8]. Nowadays, asparaginase for medical applications is only prepared from *Escherichia coli*and *Erwinia carotovora*[9, 10]. L-Asparaginase activity also has been reported from wide variety of microorganisms [10-13] and researchers are investigating these organisms to find a better and more efficient asparaginase for treatment of patients.

Although various screeningand enzyme assay techniques have been developed for L-asparaginase quantification in microorganisms, the measurement of intracellular asparaginase in bacteria is a time consuming and labor work because of steps for sonication or enzymatic disruption of bacterial cell wall. Also, due to the harsh condition of sonication, the enzyme may be inactivated during the process and the measured activity is not valid enough in point of precision and reproducibility. This is case especially for production optimization process, and scale up projects.

Hence, it is desirable to use ameasurement method for asparaginase activity which bypass the cell disruption and enzyme extraction steps. In the present study, a simple, precise and reproducible method has been validated for measurement of intracellular asparaginase activity in two strains of bacteria.

2. Materials and Methods

2.1. Strains and Culture Condition

E.coli ATCC 8739 was kindly gifted by Dr.Oshaghi (IUMS) and *Halomonas H28* was isolated from salt marsh of Iran in last study [14]. The 50 mL of overnight *E. coli*culture was used to inoculatea 500 mL LB medium in shake flask. LB medium with 10g/L asparagine was used for bacteria cultivation, except *Halomonas H28* which had an extra 1.5 M NaCl in medium. Incubation condition for both strains was set at 37°C and 180 rpm shaking for overnight.

2.2. Enzyme Extraction and L-Asparaginase Assay

After overnight incubation, the bacterial cells were harvested from medium by centrifugation at 8,000×g for 10 min. The cell pellet was washed twice by Tris-HCl buffer (50 mM, pH 8) and serially diluted in 2-fold order by the same buffer at 5 different

concentrations. After this, the cells from each concentrations were harvested in 1.5 mL micro tube by centrifugation (8,000×g for 10 min) and cells pellets were resuspended in 300 µL Tris-HCl buffer (50 mM, pH 8) for sonication and 300 µL Bug Buster lysis buffer (Invitrogen) for enzymatic lyses. In suspension method, the cells pellet was simply resuspended in 300 µL Tris-HCl buffer (50 mM, pH 8).

In sonication method, cell disruption was carried out on ice with 30 s pulse and 15 s pauses for total of 5 run using probe ultrasonic (Sonics, VC 130). Enzymatic cell disruption performed in room temperature by incubation of microtube containing cells and bugbuster reagent on 50 rpm rotator for 10 minute. Prolonged time for sonication and enzymatic incubation has been tested for increasing the recovery of enzyme (Data not include). After sonication and enzymatic treatment of bacteria, cell debris were removed by centrifugation at 16000 ×g for 20 minute and the supernatant fluid was used for assay of asparaginase activity by Nessler‘s method [15].

In cell suspension method, simply the cell suspension without any extra procedure is used instead of bacterial cell extract in Nessler‘s method.

2.3 Statistical Aanalysis

The linearity of each method was at least studied in 5 different concentrations by linear regression method. All tests were done on two species of gram negative bacteria and three replications. To evaluate the equality of the cell suspension method, for measuring the bacterial asparaginase, by conventional methods, which use ultrasonic waves and chemical and enzymatic treatments for cell disruption and enzyme extraction, these methods were compared by linear regression, Passing-Bablok regression and Bland-Altman difference plot methods [16, 17].

3. Results and Discussion

In most projects in which bacterial species are used to produce a specific product, one of the most difficult steps is screening and product optimization. At these processes, in order to identify the best producing species or the best manufacturing condition, the product should be measured carefully. Since, these measurements often need to be repeated in large numbers, hence, measurement methods in addition to having highly precision and accuracy need to be fast enough, easy to use and economically cost benefit. Routine methods require destroying the bacterial cell wall and used the cell extract in the next steps. The most common way to do this, involves using of ultrasonic waves or use of chemical and enzymatic agents for cell disruption. Some challenges of sonication methods include noise pollution in the environment as well as the use of special equipment and also the requirement of optimization of intensity and duration of sonication in different cell concentrations. Also, due to the heat generated during the process, sonication can lead to protein denaturation and lose of enzyme activity.

The enzymatic method, in addition to being time-consuming (because incubation

time with lysis buffer), is more expensive than other methods and also requires the addition of foreign substance (lysis buffer) to the cell suspension which is remained in cell extract and may be interfered with the assay in next steps. According to the above-mentioned problems, development and validation of an alternative method that bypassed the cell disruption steps can facilitate the whole project and lead to the saving of time and cost.

Common methods measuring asparaginase activity of bacteria are usually using the Nessler's reagent. In these methods, after the destruction of the bacterial cell wall, the cell extract is incubated with substrate (asparagine). In appropriate conditions (incubation time, temperature and buffer type and concentration), the bacterial asparaginase hydrolyzes asparagine into aspartic acid and ammonia. The released ammonia reacts with the Nessler's reagent and produces a colored agent that its OD in 436nm represents the asparaginase activity in bacteria.

In the suspension method, the first step (destruction of bacterial cell wall) has been bypassed and bacterial cell suspension has been incubated instead of cell extract with the substrate. The hypothesis is that during the incubation, substrate (asparagine) to penetrate inside the bacterial cells, where the bacterial asparaginase hydrolyzes the asparagine and then the released ammonia return to the reaction medium.

It has been shown that the cell suspension method, in the process of measuring intracellular bacterial asparaginase, is an acceptable alternative to the sonication method, and in the scope of this study, both methods show similar results. Also, it has been shown that the enzymatic lyses method in comparison with the sonication and the cell suspension methods have significantly higher efficiency. The results of this study were greatly consistent with the hypothesis. The results of sonication and cell suspension methods were significantly similar. But the enzymatic disruption method showed higher levels of enzyme activity in the same condition. It seems that the lower retrieval of asparaginase activity in sonication and cell suspension methods is related to some destruction of enzyme activity during the sonication process and the lack of complete access to intracellular enzymes or completes

Table 1. Test of linearity by linear regression method. Each method was assayed in *Halomonas H28* and *E.coli* strain. Tests were performed by SPSS 20. R^2 and P were presented for each methods and bacteria.

	Halomonas H28		***E.coli***	
	R^2	P	R^2	P
Sonication	0.914	> 0.001	0.900	> 0.001
Enzymatic	0.906	> 0.001	0.908	> 0.001
Cell Suspension	0.920	> 0.001	0.902	> 0.001

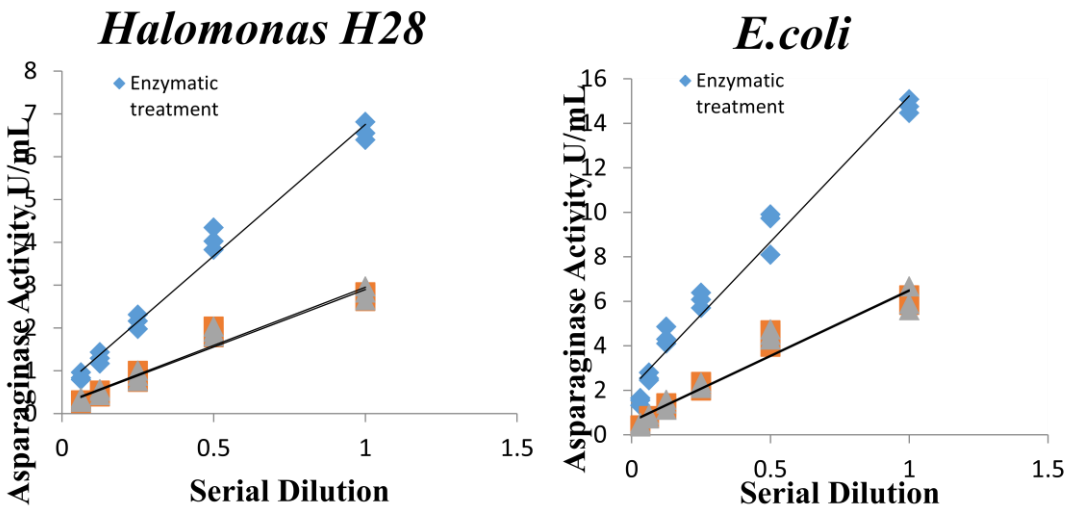

Figure 1. Analysis of linearity of all three methods in *Halomonas H28*and *E.coli* by Linear Regression.

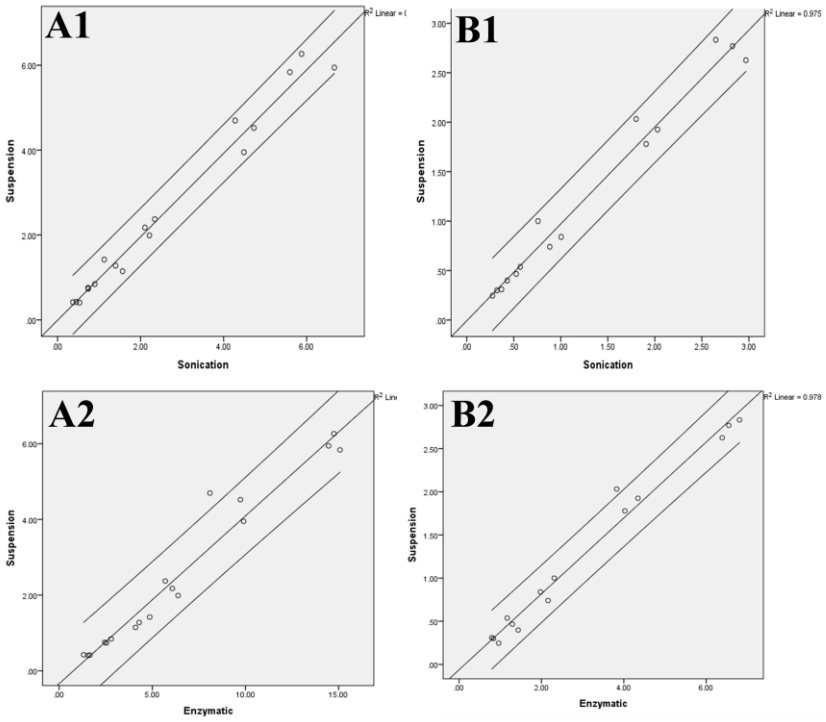

Figure 2. Comparison of methods by Linear Regression. A1 and A2. Comparison of sonication and enzymatic methods with Cell Suspension method in *E.coli*. B1 and B2 of sonication and enzymatic methods with Cell Suspension method in *Halomonas H28*.

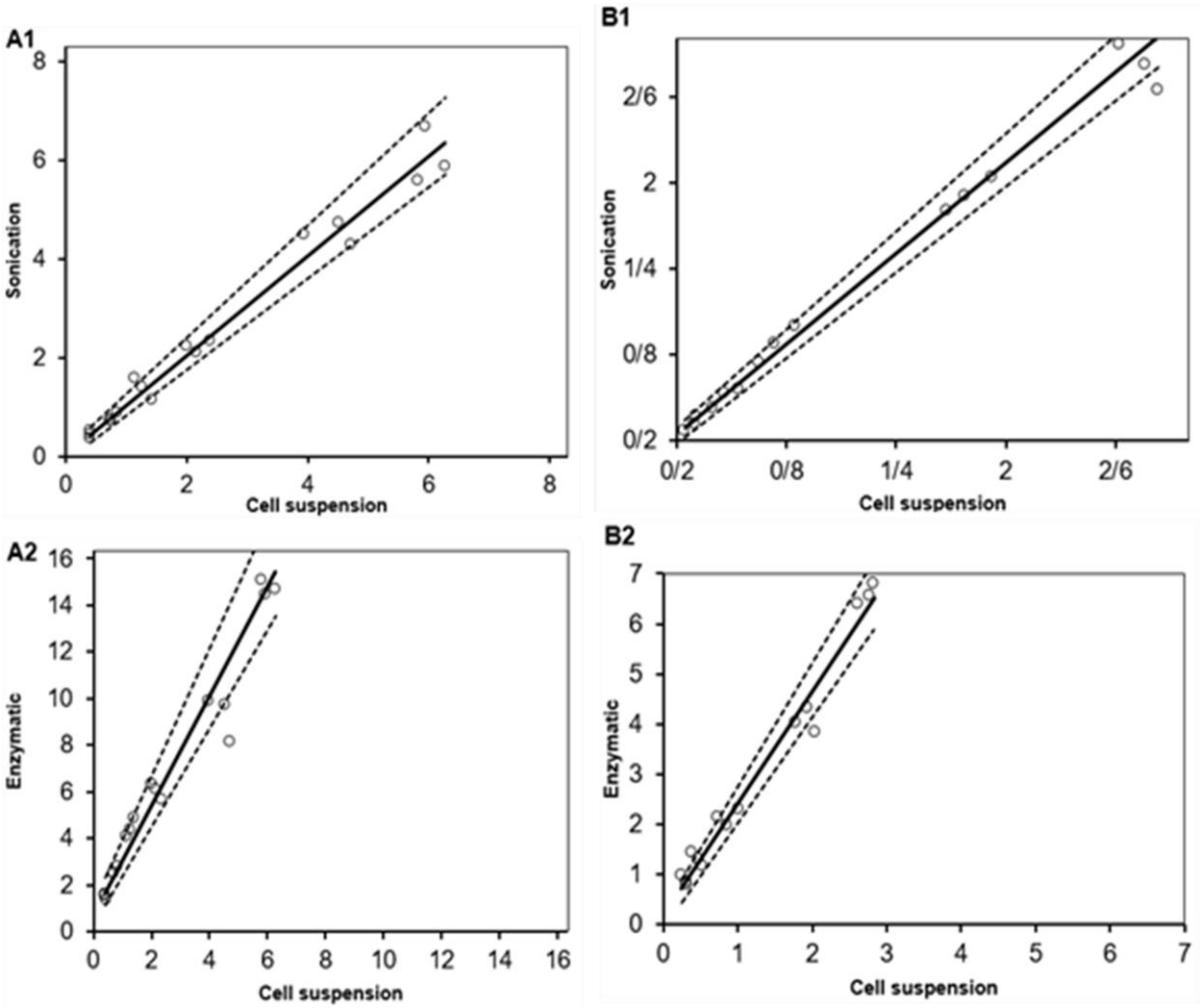

Figure 3. Comparison of methods by Passing-Bablok regression method. **A**1. Comparison of Sonication and Cell Suspension methods in *Halomonas H28.* The slop of regression line is (solid line) 1.0503 with lower and upper 95% confidence interval (dashed line) of 0.9984 and 1.1389 respectively. Also the intercept of regression line is 0.037 with lower and upper 95% confidence interval of -0.023 and 0.061 respectively. The 95% confidence interval of slop and intercept encompass the 1 and 0 respectively which mean that these two methods have shown equality for measurement of asparaginase activity. **A2**. Comparison of Enzymatic and Cell Suspension methods in *Halomonas H28.* The slop of regression line is (solid line) 2.2415 with lower and upper 95% confidence interval (dashed line) of 2.1066 and 2.4574 respectively. The intercept of regression line is 0.162 with lower and upper 95% confidence interval of -0.089 and 0.286 respectively. However the 95% confidence interval of intercept encompasses the 0, the 95% confidence interval of slop does not encompass the 1; that mean, these two methods have not shown equality for measurement of asparaginase activity. **B1.** Comparison of Sonication and Cell Suspension methods in *E.coli*. The slop of regression line is (solid line) 1.0121 with lower and upper 95% confidence interval (dashed line) of 0.9281 and 1.1391 respectively. Also the intercept of regression line is 0.011 with lower and upper 95% confidence interval of -0.104 and 0.126 respectively. The 95% confidence interval of slop and intercept encompass the 1 and 0 respectively which mean that these two methods have shown equality for measurement of asparaginase activity. **B2**. Comparison of Enzymatic and Cell Suspension methods in *E.coli*. The slop of regression line is (solid line) 2.3548 with lower and upper 95% confidence interval (dashed line) of 2.1227 and 2.7434 respectively. Also the intercept of regression line is 0.670 with lower and upper 95% confidence interval of 0.267 and 1.222 respectively. The 95% confidence interval of slop and intercept do not encompass the 1 and 0 respectively. These two methods have not shown equality for measurement of asparaginase activity.

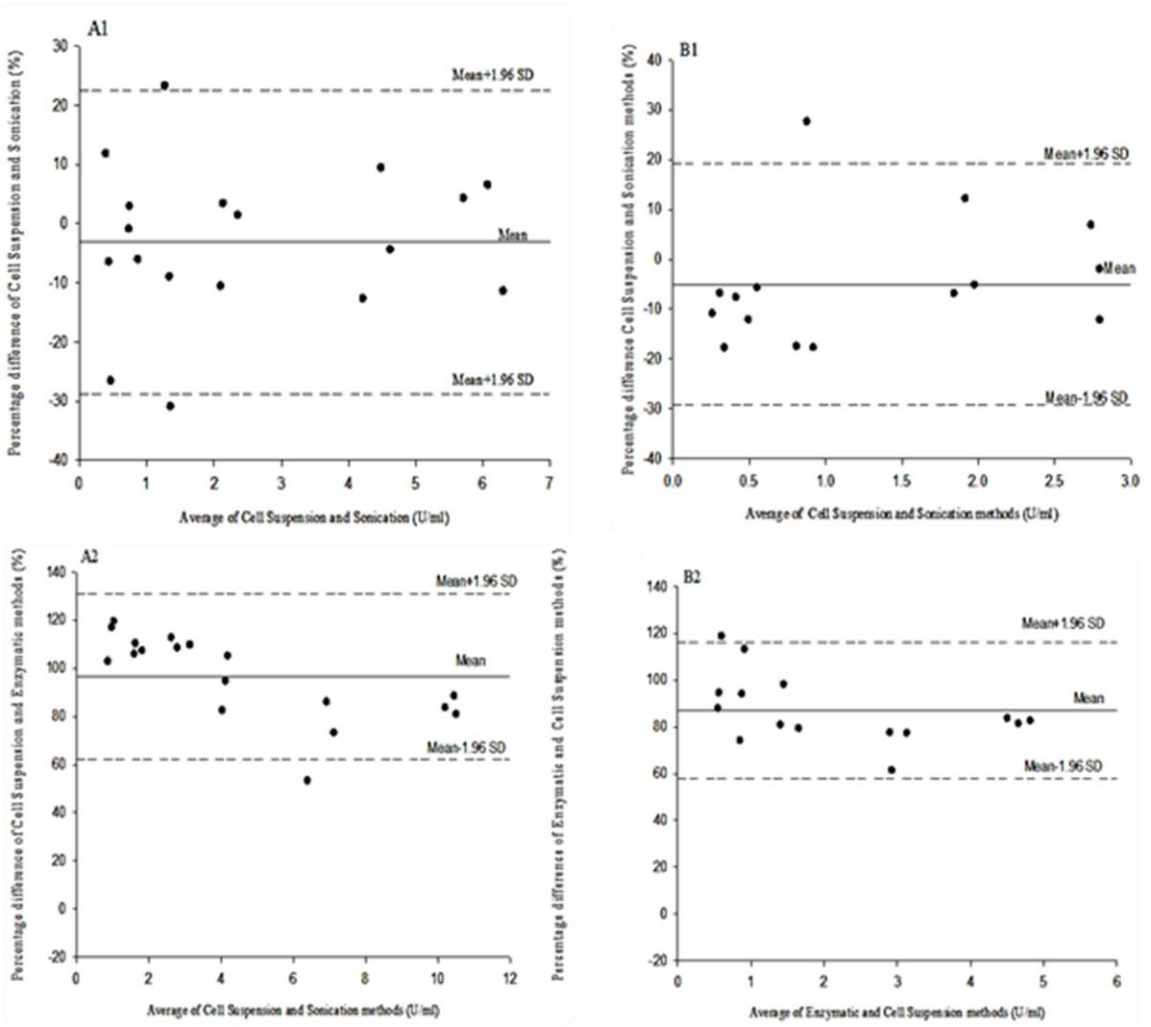

Figure 4. Bland-Altman Relative difference plot examination for calculating percentage difference between methods.**A1** and **B1**. Comparison of Cell Suspension and Sonication methods in *E.coli* and *Halomonas H28*respectively. 95% confidence interval of percentage difference of two methods encompass 0 and shown there is not statistically significance difference between two methods (P>0.05). **A2** and **B2**. Comparison of Cell Suspension and Enzymatic methods in *E.coli* and *Halomonas H28* respectively. 95% confidence interval of percentage difference of two methods do not encompass 0 and shown a statistically significance difference between two methods (P <0.001).

withdrawal of the ammonia from bacterial cells in cell suspension process.

The results have shown that in both studied bacteria (*E.coli* and *Halomonas H28*), changes in enzyme activities are in a linear relationship with bacteria cell dilution in all the three methods (Figure1 and Table 1). Most of the asparaginase activity measurements have been observed in enzymatic method, but sonication and cell suspension methods in whole range of this experiment (cell concentration of OD_{600}=0.25~4 and asparaginase activity of 0.2~15 U/mL) did not show significant differences (Figure 2, 3 and 4).

The concentrations of all bacterial samples were determined using measuring light absorption at a wavelength of 600 nm. The lowest concentration sample (1/16 dilution of initial concentration) in this study has an average of OD_{600} of 0.25, and the highest

concentration sample (without dilution) has an average OD_{600} of 4. Changes in enzyme activity in this range, in all three methods were linear, and there was no significant difference between the sonication and cell suspension methods (P>0.001). However, the enzymatic method has shown significantly higher activity of asparaginase compared to two other methods.

4. Conclusion

It can be concluded that in the measurement process of intracellular asparaginase activity of *E.coli* and *Halomonas H28* species, the cell suspension method is an appropriate alternative to sonication method. This approach, especially in production optimization projects, there are benefits from some advantages such as simplifying the procedure and reducing noise pollution, as well as saving time and costs of the procedure. Moreover, in cases where there is no restriction for use, enzymatic method compared with two other methods has shown significantly better results.

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