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RESEARCH ARTICLE

Effect of Process Parameters on Immobilization of Recombinant *Escherichia coli* on Coconut Fiber

Rohaida Che Man[1*], Siti Kholijah Abdul Mudalip[1], Nor Hasmaliana Abdul Manas[2,3], Siti Zubaidah Sulaiman[1]

[1] Faculty of Chemical and Process Engineering Technology, Universiti Malaysia Pahang Al-Sultan Abdullah, Lebuh Persiaran Tun Khalil Yaakob, 26300, Gambang, Kuantan, Pahang, Malaysia
[2] School of Chemical and Energy Engineering, Faculty of Engineering, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia
[3] Institute of Bioproduct Development, Universiti Teknologi Malaysia, 81310, Skudai, Johor, Malaysia

ABSTRACT - *Escherichia coli* (*E. coli*) is the earliest and most widely used host to produce recombinant proteins, one such example being cyclodextrin glucanotransferase (CGTase) which has found application in numerous industries such as food, environmental engineering and pharmaceutical. The recombinant protein in *E. coli* was applied to overcome the low output of CGTase from *Bacillus* sp such as *Bacillus lehensis and Bacillus licheniformis*. However, cell lysis and plasmid instability have been some of the stumbling blocks during the production of recombinant protein in *E. coli*. Therefore, cell immobilization has been proposed as a way to increase CGTase production while maintaining high cell stability. The objective of this study is to immobilize recombinant *E. coli* on coconut fiber. The effect of the process parameters, namely pH level (5, 6, 7, 8, and 9), contact time (12 hr, 15 hr, 18 hr, 21 hr, 24 hr, and 27 hr), and temperature (20 °C, 25 °C, 30 °C, 35 °C, and 40 °C), towards the immobilization of recombinant *E. coli* onto coconut fiber was studied. The optimal pH resulted in the highest immobilization yield was pH 8 (58.90%). The best contact time for the highest immobilization yield (57.59%) was 24 hours. The optimum temperature was identified at 25 °C with immobilization yield of 58.78%. Hence, the optimum conditions could improve the immobilization of recombinant *E. coli* on the carrier and the coconut fibre was an appropriate carrier material for cell immobilization process for the high CGTase production with high cell stability.

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1.0 INTRODUCTION

E. coli is a bacterium that has unique characteristics, such as ease of handling and the ability to grow in both aerobic and anaerobic conditions, that make it a valuable host organism in biotechnology [1]. It is an indispensable host for the synthesis of biochemicals and enzymes. *E. coli* is employed in a variety of industries, including medications, food ingredients, and pigment.

An industrial enzyme namely cyclodextrin glucanotransferase (CGTase) catalyze the starch to produce cyclodextrin [2]. Most CGTase are produced by bacteria and predominantly by *Bacillus* species. It is a common industrial enzyme found in the plastics, food, pharmaceutical and cosmetics industries [3]. Industrial production of cyclodextrin demands a correspondingly high output of CGTase, but *Bacillus* sp. output is incapable of keeping up with this demand. In this study, the CGTase gene that was utilized formerly isolated from the *Bacillus lehensis* G1 genome [4]. The low production of CGTase (20.30 U/ml) was obtained from this microbial. Hence, the recombinant *Bacillus* CGTase production in *E. coli* was performed by Ong et al [5], whereas the high CGTase production was detected. Nevertheless, the expressed recombinant CGTase in *E. coli* by using native signal peptide was formed the inclusion bodies with only 35.71 U/ml of CGTase activity was obtained. The M5 was used as a mutated signal peptide to solve the problem in inclusion bodies formation [6]. The production of CGTase was improved up to 90 U/ml (2.5- fold) by using M5 in comparison to native signal peptide, but the level of cell lysis was similar. Thus, cell immobilization is used to address the difficulties due to its ability to increase the CGTase production with low cell lysis [7–9]. The immobilization technique offers the best condition for the growth and production of enzyme. The cells can grow as a group of colonies by selecting an appropriate condition for growth. Thus, the cells were preserved from environmental stress and reduced the occurrence of cell lysis. Moreover, the cells attachment on the surface of carrier have frequently been correlated to the development of biofilms. These biofilms binding the cells permanently to each other and to the carrier surface following an initial reversible adsorption [10]. Therefore, the biofilms serve the best place for the plasmids exchange and preservation of plasmid, resulting in high plasmid stability and effective production of CGTase.

The cell immobilization process is the alternative method to enzyme immobilization. Cell immobilization confines high cell loading into or onto a carrier, which can be charged into a bioreactor for the production of high value chemical compounds [11]. Cell immobilization are generally inexpensive procedures, allow for cell reuse, have low bulk viscosity,

***CORRESPONDING AUTHOR | R. C. Man** | ✉ rohaida@umpsa.edu.my

high stability, and tolerance to pH and temperature fluctuations or swings [12,13]. Cell immobilization may be achieved through a variety of methods, such as entrapment, encapsulation, cross-linking, and adsorption. These methods have been used to increase the cell loading and yield of the desired product. For example, a study performed by Ashikin et al. [14] on recombinant *E. coli* adsorbed onto graphene oxide found increased xylopentaose sugar yield. Moreover, highly thermostable endoxylanase production, reduction of cell lysis and cell plasmid stability were observed in this study as well. Vassileva et al. [15] showed that the entrapment of *B. circulans* ATCC 21783 using an agar gel in a fluidized bed reactor increased the CGTase production up to 180 U/ml-210 U/ml of enzyme activity after 24 hours and 48 hours of cultivation. Thus, careful consideration of the cell immobilization technique is important to improve the cell immobilization yield and desired product. The recombinant *E. coli* was immobilized onto coconut fiber via the adsorption technique in this present study. Adsorption has advantages over other methods due to the simple method during the cell immobilization process and possibly lower mass transfer limitation between the cells and carrier [16].

Numerous works have been performed to study cell immobilization using diverse carriers such as charcoal, cation exchanger, hollow fiber membrane, and alginate [9,17–19]. The material of carrier is divided into two categories, namely organic (natural and synthetic compounds) and inorganic materials. Both categories are commonly used to immobilize different types of cells. As example for organic carrier, Ercole et al. [20] showed that *Actinobacillus succinogenes* produced more succinic acid when it was immobilized in alginate beads versus when it was free-floating. The biocatalyst was also successfully operated up to 3 months and it can be reused without impaired the performances. Meanwhile, the inorganic carriers such as silica, porous glass and metal oxide are capable to provide high mechanical and thermal strength for cell immobilization. Zhao et al. [21] demonstrated that *Lactobacillus rhamnosus* immobilized on an inorganic carrier (mesoporous silica) achieved a high conversion of glucose into lactic acid (92.4%), and the yield did not diminish even after the immobilized had been reused eight times.

The carrier used in the present study for cell immobilization was untreated coconut fiber. The problems that always related to the immobilization carriers are high cost, swelling and poor mechanical stability. As an agricultural waste, coconut fiber is rigid and it is the strongest among all natural fibers, in addition to being able to extend its length by up to 6 times higher compared to other fibers [22]. The fiber contained of tannins, pectin, flavonoids, polyphenols, cellulosic, hemicelluloses, and lignin as binding substance [23, 24]. Moreover, coconut fiber is cheap and environmentally benign. Therefore, based on these characteristics and structures of coconut fiber, it can be potentially used as a carrier for cell immobilization. A high immobilization yield with simple separation of cells during fermentation would be possible by using the coconut fiber as the carrier.

The aim of this work is to immobilize recombinant *E. coli* on coconut fibre. The influence of process parameters like pH, contact time and temperature on the recombinant *E. coli* immobilization using coconut fiber was investigated. These parameters could influence the cells growth and cells immobilization process. To date, there has been no research works on recombinant *E. coli* immobilization using coconut fiber as a carrier. Most studies for the immobilization of *E. coli* thus far have used other carriers such as hollow fiber membrane [13], microplate [25], carbon nanotube [7], charcoal [18], and graphene oxide [14]. The findings presented here suggest that the optimum temperature, contact time and pH could enhance the immobilization of *E. coli* on the coconut fiber. Coconut fiber was also identified as a significant material to be applied as a carrier for the cell immobilization.

2.0 MATERIALS AND METHODS

2.1 Materials

Coconut fiber was gained from Nirwana Sdn Bhd (Kuantan, Malaysia). Citric acid, monobasic sodium phosphate, sodium hydroxide, dibasic sodium phosphate and glycine were bought from Merck Sdn Bhd (Selangor, Malaysia). Tryptone, glycerol, sodium chloride, yeast extract, ampicillin, magnesium chloride, potassium chloride, phenolphthalein, and sodium carbonate were bought from Friendemann Schmidt, Parkwood, Australia.

2.2 Bacterial Strain

The construction of the strain for recombinant *E. coli* producing CGTase from *Bacillus lehensis* G1 was performed following the method by Jonet et al. [6]. *E. coli* JM109 was used as a host for cloning, whereas *E. coli* BL21 (DE3) was used for the expression process. For cloning purposes, the pET-21a (+) (Novagen) was implemented as the vector backbone. An antibiotic used was ampicillin (100 µg/ml). The cells were induced using 0.01 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) as an inducer.

2.3 Cell Immobilization on Coconut Fiber

Before the process of cell immobilization, the coconut fiber was cleaned and washed to remove contaminants and impurities and then it was dried at 90 ℃. Coconut fiber was chopped to size of 3 cm x 3 cm x 3 cm (length x width x thickness) and sterilized for 15 min at 121 ℃. In a 250-ml flask, the coconut fiber and 50 ml of Luria Bertani, (LB, 10 g/l tryptone, 5 g/l yeast extract, and 5 g/l NaCl) broth with ampicillin at the concentration of 100 µg/ml were mixed together. The 250-ml flask was inoculated with the cells from the glycerol stock and incubated using 25 °C of temperature and 200 rpm of agitation rate. The coconut fibre was taken out and rinsed thoroughly with sterile distilled water to wash away any loose cells after 24 hours of incubation.

2.4 Effect of Process Parameters on Recombinant E. coli Immobilization

2.4.1 Effect of pH

The influence of pH on the recombinant *E. coli* immobilization on the coconut fiber was investigated at different pH levels: 5, 6, 7, 8, and 9 [7, 22]. In this study, phosphate-citrate buffer was used to maintain pH 5 and pH 6 in the medium, whereas sodium phosphate buffer was used to maintain pH 7 and pH 8. Meanwhile, glycine-NaOH buffer was used for pH 9. The cell immobilization process was performed at different pH levels, while maintaining agitation at 200 rpm, temperature at 25 ℃ and incubation time at 24 hours for all runs. Then, the immobilization yield or cell concentration on the carrier was calculated using Equation (1). All values of immobilization yield were calculated from the average of at least three replicates.

$$X\ (\%) = \frac{(W_0 - W_1)}{W_1} \times 100\% \quad (1)$$

where X is the immobilization yield, W_0 is the weight of dry coconut fiber and dry cells (mg) and W_1 is the dry coconut fiber weight (mg).

2.4.2 Effect of Contact Time

The influence of contact time on the recombinant *E. coli* immobilization on coconut fiber was investigated by performing the immobilization process with varying contact time: 12 hours, 15 hours, 18 hours, 21 hours, 24 hours, and 27 hours [26], while maintaining pH 8, 25 ℃ and agitation at 200 rpm for all runs. The immobilization yield was calculated using Equation 1.

2.4.3 Effect of Temperature

The influence of temperature on the recombinant *E. coli* immobilization using coconut fiber as a carrier was studied by performing the immobilization at varying temperatures: 20 ℃, 25 ℃, 30 ℃, 35 ℃, and 40 ℃ [5, 9], while maintaining agitation at 200 rpm**,** 24 hours of incubation, and pH 8. Immobilization yield was calculated by using Equation 1.

2.5 Analytical Methods

2.5.1 Immobilization Yield (Cell Concentration)

Before the immobilization process, the coconut fiber was dried at 90 °C and sterilized at 121 ℃ for 15 minutes, then weighed. After the cell immobilization process, the coconut fibre was dried until constant weight. Immobilization yield (cell concentration) was calculated using Equation 1.

2.5.2 Scanning Electron Microscope (SEM)

After the immobilization process, the coconut fiber was prepared for scanning electron microscopy (SEM). For fixation, the coconut fiber was immersed overnight in 2.5% glutaraldehyde solution at 4 °C. Then, the coconut fiber was rinsed with distilled water and dehydrated by immersing in progressively drier ethanol solutions (50%, 70%, 80%, 95% and 100%), for 10 minutes in each concentration, followed by drying at the critical point. Then, it was coated with gold and SEM photographs were taken by using HITACHI TM3030.

3.0 RESULTS AND DISCUSSION

3.1 Effect of pH on Immobilization Yield

One of the important parameters that affecting the cell immobilization process is pH. The influence of pH on the immobilization yield was investigated by conducting the process with the medium at pH 5, 6, 7, 8, and 9, while maintaining the temperature at 25 °C, incubation for 24 hours and agitation at 200 rpm. The highest immobilization yield of 58.90% was obtained at pH 8, followed by 52.06% at pH 7 as shown in Figure 1. The immobilized yield increased steadily with increasing pH from 5 to 8. Since adsorption relies on the surface properties of both the carrier and the subject to form the attachment, particularly surface charge and hydrophilicity, these factors must be considered to obtain the most efficient cell immobilization process on a carrier. Normally, the outer coating of bacterial cells, including *E. coli* have a net negative charge and are hydrophobic [27–29]. Meanwhile, the coconut fiber behaves as a hydrophobic carrier [30]. Thus, one probable mechanism responsible for the effective adsorption of recombinant *E. coli* onto the coconut fiber is the hydrophobic interactions [29]. Another possibility is an electrostatic interaction between the anions (abundant OH^- at pH 8) and the cell coating's positively charged domains. The positively charged domains composed of an exclusive mixtures of proteins, carbohydrates, polysaccharides, lipid, and DNA [31,32] would contribute to the biofilm formation. The electrostatic interaction between the biofilm and the coconut fiber could form a strong attachment between cells and the coconut fiber, which would explain the high immobilization yield observed at pH 8. Man et al. [8] discovered strong interactions (a hydrophobic interaction and two electrostatic interactions) between recombinant *E. coli* and hollow fiber membrane forming at pH 9 that contributed to the favourable adsorption of recombinant *E. coli* onto the hollow fiber membrane.

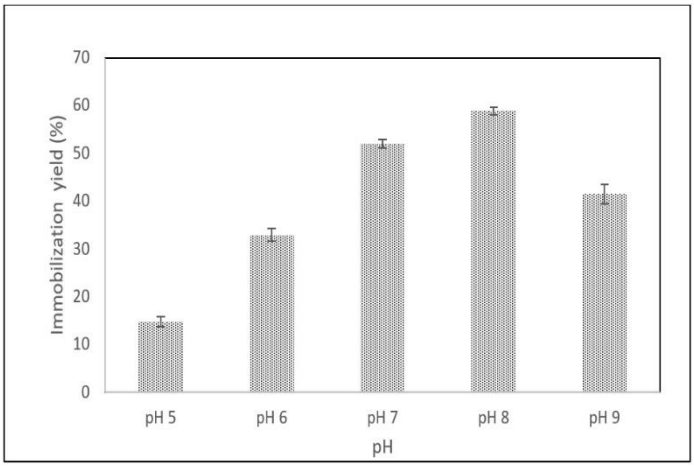

Figure 1. Effect of pH on the immobilization yield. The fermentation conditions used in this study: 24 hr, 25 ℃ and 200 rpm.

In addition to its effect on surface properties, a medium that was too acidic or too alkaline could have hindered the growth and proliferation of the bacteria by disrupting its metabolism, and thus were not favourable for the attachment of cells on the coconut fiber. Based on Figure 1, the immobilization yield was 14.78% at pH 5 and 41.54% at pH 9. A contradict result was obtained by Wang et al. [33] on the immobilization of *Bacillus circulans* ATCC 21783 using a loofa sponge as a carrier. The finding showed that the optimum pH was 9 for the immobilized *Bacillus circulans* ATCC 21783 on the loofa sponge. In summary, it can be concluded that pH 8 is optimal for the recombinant *E. coli* immobilization on coconut fiber in order to obtain the highest immobilization yield.

3.2 Effect of Contact Time on Immobilization Yield

In this present work, the immobilization process was conducted at varying contact times: 3 hours, 6 hours, 9 hours, 12 hours, and 15 hours, to investigate their effect on immobilization yield, while maintaining the medium at pH 8, 25 ℃, and agitation at 200 rpm. The highest cell immobilization yield (57.59%) was detected after 24 hours of contact time, followed by 48.46% immobilization yield detected at 21 hours of contact time as shown in Figure 2. As the culture was left in the incubator for longer time, the number of cells attached to the surface of carrier was increased. De Souza et al. [17] reported that they obtained the best yield after 15 hours of contact time for *Erwinia* sp. D12 immobilization using an algaroba gum and alginate blend. The immobilized cells were apparently highly stable for up to 72 hours and achieved high isomaltulose yield (167.52 g/l).

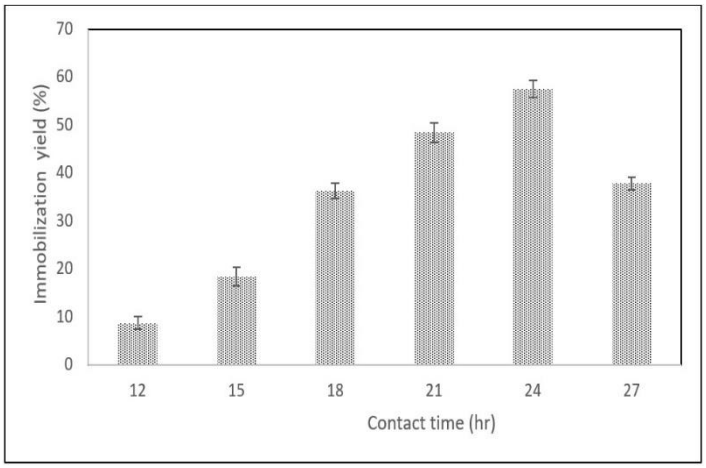

Figure 2. Influence of contact time on the cell immobilization yield. The fermentation was conducted at pH 8, 25 ℃, and 200 rpm agitation.

From Figure 2 it shows that the cell immobilization yield declined to 37.81% as the incubation was extended to 27 hours. It was found that some cells had detached themselves from carrier surface at this point in time. The detachment could be caused by the long period of submersion [34]. However, Duarte et al. [35] observed that contact time longer than 10 hours was not conducive for the stable production of ethanol by the immobilized *Saccharomyces cerevisiae* in calcium alginate bead. They surmised that the bead had begun to rupture, preventing their reuse. This result suggested that the integrity of the carrier against the medium is a factor to be considered. Thus, from the results obtained in the study, it can be concluded that to achieve the highest immobilization yield, the optimal contact time for the recombinant *E. coli* immobilized on coconut fiber was 24 hours.

3.3 Effect of Temperature on Immobilization Yield

The cell immobilization process is very sensitive to temperature. The temperature effect was explored by running the immobilization procedure at 20 ℃, 25 ℃, 30 ℃, 35 ℃, and 40 ℃, while maintaining the medium at pH 8 and incubation at 200 rpm agitation for 24 hours. Figure 3 shows that as we raised the temperature from 20 ℃ to 25 ℃, immobilization yield improved. Immobilization yield was the highest (58.78 %) at 25 ℃. Recombinant *E. coli* proliferate readily at this temperature [9], thus there were more cell-coconut fiber linkages, and subsequently higher cell immobilization yield. Figure 4 shows the micrographs of recombinant *E. coli* cells on coconut fibre at 25 ℃, where it was apparent that more cells were attached onto the coconut fiber at 25 ℃. In contrast, Chen et al. [36] found that the optimal temperature was 36 °C for the immobilization of mixed cultures on poly-(vinyl alcohol)-alginate for the production of hydrogen. The mixed culture achieved the highest cell density and the highest hydrogen throughput at this temperature which they are well adapted to.

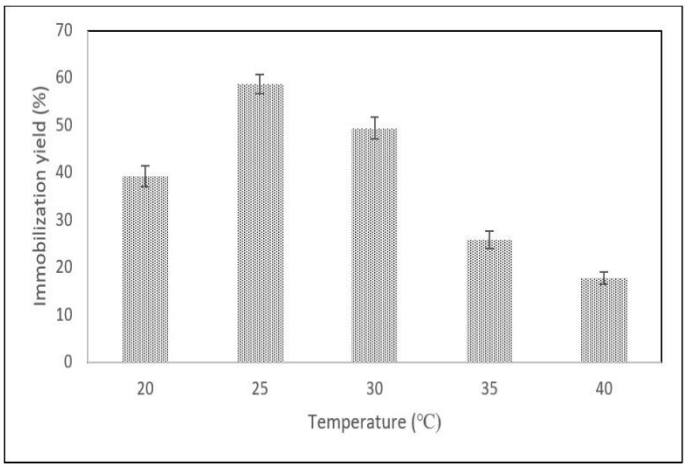

Figure 3. Effect of temperature on the recombinant *E. coli* immobilization. The process was performed at pH 8, 24 hours incubation, and 200 rpm agitation.

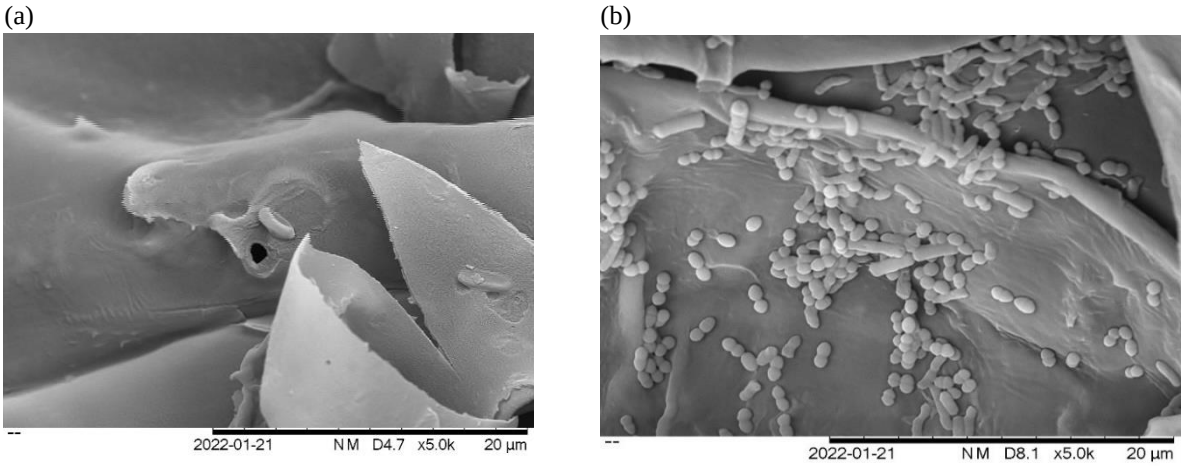

Figure 4. Micrograph of coconut fibre before (a) and after (b) cell immobilization process at x5000 magnification.

When the temperature was raised from 25 ℃ to 40 ℃, the cell immobilization yield decreased to 17.78%. The cell densities declined as more bacterium perished at such high heat [37]. This condition also impacted membrane integrity, thus hindering the cell immobilization process. The experimental evidence supported that 25 ℃ was the best temperature for the highest recombinant *E. coli* immobilization yield on the coconut fiber.

4.0 CONCLUSION

The optimal pH, contact time, and temperature used in the process of immobilizing recombinant *E. coli* on coconut fiber is very important to maximize the immobilization yield and to increase the stability of the immobilized cells. The optimum pH, contact time and temperature for the highest cell immobilization yield were pH 8, 24 hours and 25 °C, respectively. It is clear from this study that the coconut fiber is a good carrier for the cell immobilization, helping to boost CGTase production and keeping cell lysis rate low. It was an effective and easy technique; in situ cell immobilization process was implemented without pre-treatment of the coconut fiber with any reagents. In addition to that, the

immobilized cells on the coconut fiber presented to have significant potential in recombinant protein production because of the high quantity of cells that were immobilized which could result in high reusability and enhanced cell stability.

5.0 CONFLICT OF INTEREST

The authors declare no conflicts of interest.

6.0 AUTHORS CONTRIBUTION

Rohaida Che Man (Conceptualization, Visualization, Writing-original draft)

Siti Kholijah Abdul Mudalip (Conceptualization; Formal analysis)

Nor Hasmaliana Abdul Manas (Methodology; Writing-review & editing)

Siti Zubaidah Sulaiman (Writing-review & editing)

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