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Effect of Complex Nutrients and Repeated-Batch Cultivation of *Halobacterium salinarum* on enhancing Bacteriorhodopsin Production

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Abstract

The extreme halophilic bacteria *Halobacterium salinarum* is known to produce bacteriorhodopsin (BR) protein in its purple membrane (PM) as a light-driven pump for the synthesis ATP. Its growth does not utilize simple carbon sources such as glucose, but relies on the complex carbon/nitrogen sources. The production yield of BR in the culture of *H. salinarum* also strongly depends on the complex nitrogen sources employed. From the various complex carbon/nitrogen sources employed in this work, tryptone rather than commonly used peptone was found to be the best complex nutrient for the growth of *H. salinarum* and its BR production. Bubble column photobioreactor operated in a repeated-batch mode was also employed to enhance the growth of *H. salinarum* by intermittent removing growth inhibitory metabolites. By employing 0.5% tryptone as carbon/nitrogen source in the culture medium, 201.8 mg/L of BR was obtained after 210 h repeated-batch cultivation which is about 50% higher than that obtained in shaker flask cultivation.

Keywords: Bacteriorhodopsin production; Complex nutrients; Photobioreactor

Introduction

Bacteriorhodopsin (BR) is the only protein in the purple membrane (PM) patch of extreme halophilic bacteria *Halobacterium salinarum*. It functions as light-driven proton pump to transfer protons unidirectional from the cytoplasmic side to the extracellular side of the membrane. Thus, the established proton gradient will drive the synthesis of ATP for cell metabolism. Due to its unique light-driven proton pumping property and exceptional thermal and photo stability, a considerable interests in incorporating BR film into electronic circuitry for the generation of photocurrent have been reported which includes artificial retinas, photochromic data storage, holographic cameras, information processing and etc. [1-6]. In addition, BR has also been incorporated with inorganic nanoparticles to form bio hybrid nanocomposites for the preparation of bio-capacitor [7], novel photovoltaic stack system [8], and light-powered pH sensor [9]. Despite of a large quantity of BR is in demand for research and development, only a small amount is available due to the difficulty of cultivating Halobacterium and poor yield of BR production. In addition, the lengthy and tedious standard sucrose gradient ultracentrifuge (SGU) for BR purification also limited the availability of purified BR. Recently; we have solved the large-scale BR purification problem by using the easily scalable aqueous two-phase system (ATPS) for BR purification. The BR directly purified from the lysate of *H. salinarum* cells by ATPS has a comparable purity as that obtained from the traditional SGU purification process [10]. Therefore, the remaining problems of low production yield of BR and difficulty of cultivating halo bacterium need to be solved before the high purity BR becomes affordable and available for the demand of for research and development.

H. salinarum has been grown in a continuous culture for studying the regulation of BR synthesis by growth rate [11]. Most often it was cultured in batch mode for BR production, and cell density obtained was quite low (OD660=1 ~ 2) in 3–5 days [12,13]. In order to enhance the cell density, cell recycle cultivation has been tried by Lee et al. [14] using a bioreactor equipped with an external hollow fiber membrane unit. In addition to the cultivation operational strategy, the effect of various complex carbon/nitrogen sources and amino acids on the growth of *H. halobium* has been studied [15]. Besides, the studies on optimizing culture media components for the growth of *H. salinarum* and BR production have also been reported [16,17].

Peptone is a commonly used nitrogen sources for the cultivation of various microorganisms. It is mainly a mixture of polypeptides and amino acids produced by acidic or enzymatic hydrolysis of animal or plant proteins. However, it has been confirmed that *H. salinarium* does not utilize simple carbon sources such as glucose and sucrose; in contrast its growth relies on the complex carbon/nitrogen sources such as yeast extract and peptone [15-17]. Since peptone can be generated from different proteins sources via different preparation methods, to the best of our knowledge no study on the effect of different peptones on the growth of *H. salinarum* for BR production has been reported. Therefore, in this study peptones produced from meat, soybean, and milk as well as yeast extract were employed to investigate their effects on the growth of *H. salinarum* and BR production. In order to enhance the productivity of *H. salinarum* for BR, the repeated-batch cultivation was also employed in a bubble column photobioreactor to reduce the accumulation of metabolite inhibitors and skip the long lag phase encountered in batch operation.

Material and Methods

Chemicals

Peptone bacteriological (Oxoid code: LP0037) was purchased from Thermo Fisher Scientific and tryptone (Bacto Cat. 211705), yeast extract (Bacto Cat. 212750) and soytone (Bacto Cat. 243620) were

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purchased from Becton, Dickinson and Company. All other chemicals used are analytical grade.

Culture conditions

Halobacterium salinarum strain obtained from Professor Dieter Oesterhelt (Max Planck Institute of Biochemistry, Martinsried) was maintained on agar plates prepared by dissolving 25 g NaCl, 2 g $MgSO_4 \cdot H_2O$, 0.3 g sodium citrate, 0.2 g KCl, 1 g bacteriological peptone and 2 g agar (Difco) in 100 mL deionized water (18.2 $M\Omega cm^{-1}$) with pH adjusted to 7.2 using 1 M NaOH. The same medium but without agar was also used for liquid culture. A single colony selected from the agar plate was inoculated into 100 mL culture medium in a 250 mL shaking flask and cultivated at 37°C with150 rpm under continuous illumination (40 W). After 48 h cultivation, the optical density (OD) of cells at 660 nm was about 1.5 and used as a seed culture for the bubble column photo reactor cultivation.

Effect of complex nitrogen sources on *H. salinarum* cultivation

Bacteriological peptone, tryptone, and yeast extract were used as complex nitrogen/carbon sources to study their effect on the growth of *H. salinarum* and BR production. The cell pellet was collected from a 100 mL seed culture of OD_{660} ~ 1.5 by centrifugation (Beckman Avant J25, rotor JA10) at 8000 rpm and 4°C for 15 min. The collected pellet was re-suspended in 100 mL fresh culture medium containing different nitrogen sources to a cell concentration about OD_{660} 0.15. The cell was cultivated in a 250 mL shaking flask at 37°C with 150 rpm under continuous illumination (40 W). Low oxygen supply was implemented to induce BR synthesis by covering the mouth of shaker flask securely with a Para film which was punctured to allow small amount of air exchange [18]. Cell growth was measured by OD_{660} and the dried cell concentration was calculated by using the correlation equation of y=1.1991x where x is OD_{660} and y is dried cell weight (g) per culture volume (L).

Repeated-batch cultivation in a bubble column photobioreactor

The cell growth in batch culture of shaking flask was compared with that in bubble column photobioreactor. The total volume of photobioreactor is about 1 L and the working volume used is 800 mL. The diameter of outer column is 10 cm and the diameter of inner glass column for installation of LED lamp is 2.2 cm. The height of column is 15 cm. The air is supplied from bottom of the column to the annular area to generate bubbles and also provide mixing. The reactor was surrounded by 4 fluorescent lamps (40 W) and 1 LED lamp strip inserted in a glass tube which installed at center of the bioreactor for illumination. For shaking flask cultivation, in a 1 L shaker flask 400 mL tryptone culture medium was employed, seed culture was inoculated to a concentration about OD_{660} 0.15 to start the cultivation. For the cultivation carried out in bubble column photobioreactor, 800 mL half-tryptone medium was employed in which tryptone concentration was reduced from 1% to 0.5%. The photobioreactor is situated in a 37°C water bath and the reactor was stirred with a magnetic stir bar at 50 rpm. Aeration rate was set at 1 vvm for 48 h then decreased to 0.1 vvm for reducing low oxygen supply until the end of cultivation. For repeated-batch culture, the cell pellets were collected by centrifugation (Beckman Avant J25, rotor JA10) with 8000 rpm at 4°C for 15 min and re-suspended with same volume of fresh tryptone medium for shaker flask cultivation and half-reduced tryptone concentration medium for bubble column photobioreactor, respectively.

Bacteriorhodopsin quantification

In a typical BR quantifying operation, cell pellets in 2.9 mL culture of OD_{660}0.2 were harvested by centrifugation (Eppendorf centrifuge, rotor FA-45-24-11) with 8000 rpm for 15 min. The collected cell pellets were resuspended by 0.6 mL deionized water containing 0.9 µL DNase solution (1.2 U, Sigma-Aldrich). After well-mixing for 30 min at room temperature, the cell lysate solution was obtained. The cell lysate was centrifuged at 24000 rpm (Eppendorf centrifuge, rotor FA-45-24-11) for 30 min at 4°C to obtain the purple pellets. After washing the purple pellets twice with 0.6 mL deionized water, the purple pellets were resuspended in 0.6 mL deionized water. The absorbance at 568 nm of the solution was immediately measured after 60 µL pH 7, 2 M hydroxylamine solution was added. The bleaching reaction of BR with hydroxylamine was carried out for 24 h under illumination (40 W). The 24 h absorbance difference was employed to calculate BR concentration based on molar extinction coefficient of 63,000 $M^{-1}cm^{-1}$ [18].

Bacteriorhodopsin concentration (mg/L culture) = 26800 × $\frac{A_{568}^{0} - A_{568}^{24}}{63000}$

Where 26,800 is the molecular weight of BR.

To compare the data obtained from different culture medium for determining their statistical probability of differentiation, one-way ANOVA method in Origin software was employed. When $p<0.05$, the two sets of data were distinguishable. When $p>0.05$, the two sets were not statistically distinguishable.

Result and Discussion

Effect of peptones and yeast extract as complex medium on cell growth and BR production

Complex nitrogen sources bacteriological peptone, tryptone, soytone, and yeast extract are hydrolysis products derived from meat, milk, soybean, and yeast, respectively. Their effects on growing *H. salinarum* and BR production were first studied in shaker flask culture. Since soytone cannot be completely dissolved in the basal medium containing very high NaCl concentration (25%, w/v) required for the growth of halophilic *H. salinarum*, soytone was abandoned for the comparative study. In order to induce BR synthesis, low oxygen cultivation was adopted at the end of exponential growth phase. As shown in Figure 1, *H. salinarum* had a very long growth lag (~ 14 h) in all the cultures but started to grow much faster in yeast extract medium than in bacteriological peptone and tryptone cultures. Stationary phase was reached at 46 h in yeast extract culture. The highest cell concentration (3.8 g/L) achieved in yeast extract culture was 2 and 1.8 fold of peptone and tryptone medium cultures, respectively. According to the specification provided by suppliers, the total nitrogen content of yeast extract (10.9%, w/w) is lower than the other two peptones (15.2%, w/w for peptone and 13.3% for tryptone) but with highest content of amino nitrogen (6% w/w vs 5.3% for tryptone, 2.9% for peptone). Besides, yeast extract is known to have a richer content of vitamin B complexes and growth factors in comparison with peptones. Probably, the higher amino nitrogen content and the presence of vitamin B complexes and/or growth factors leads to the high cell growth rate and cell density in yeast extract culture. When low oxygen supply was implemented to induce BR synthesis at 46 h, the cells stopped growing and stationary phase was reached (Figure 1). The color of final yeast extract culture (inset of Figure 1) was also very different from expected

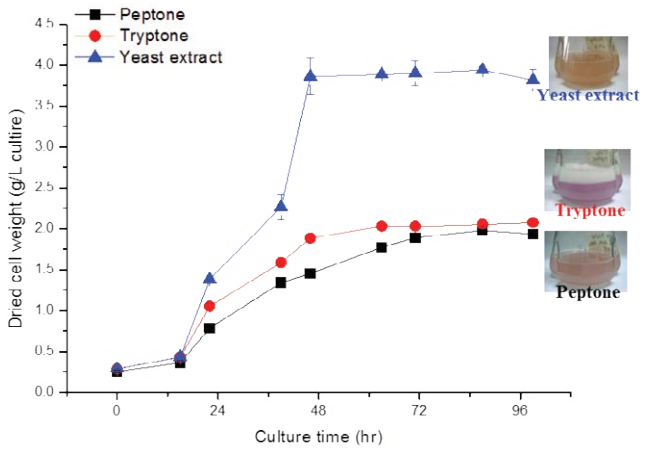

Figure 1: Growth curves of *H. salinarum* cultivated in bacteriological peptone, tryptone and yeast extract medium.

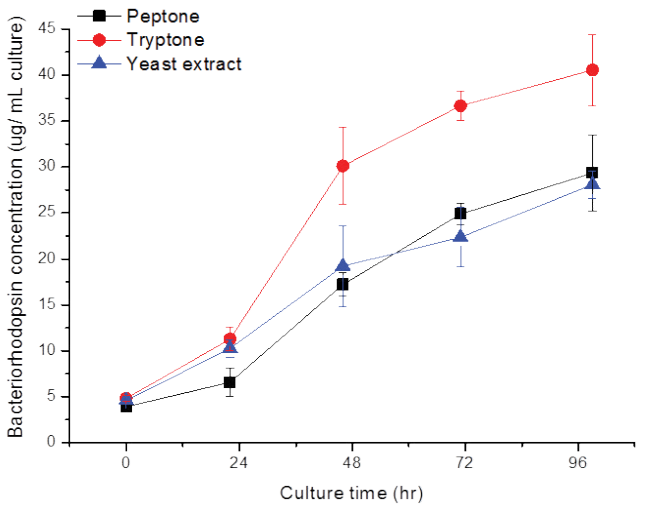

Figure 2: Bacteriorhodopsin produced by *H. salinarum* cultivated in bacteriological peptone, tryptone and yeast extract medium.

purple color which indicates the presence of purple membrane as shown in the other two cultures.

The amount of BR produced in the culture with respect to culture time was shown in Figure 2. In the tryptone medium, BR concentration increased with time to 40.6 mg/L at 96 h. It is approximately 1.4 fold higher than that obtained in yeast extract and peptone cultures. Evidently, *H. salinarum* grew slower and resulted in a lower cell concentration in tryptone culture but achieved a higher BR concentration than other two cultures. This indicates *H. salinarum* cells grew in tryptone culture is more productive for BR. The BR content of the cells was also calculated based on the obtained dried cell weight. As shown in Figure 3, BR contents of 19.5, 15.2 and 7.4 mg/g were obtained for cells collected from tryptone culture at 96 h, peptone culture at 99 h and yeast extract culture at 96 h, respectively. The photo image (inset of Figure 3) of the cell pellets collected from tryptone culture also confirmed that tryptone medium is better for BR production due to the deepest purple color achieved. The cell concentration of tryptone culture was similar to that of peptone culture but with a higher BR productivity, probably 5.3% amino nitrogen in the tryptone provided *H. salinarum* cells enough nutrient sources to synthesize BR in contrast with 2.9 % amino nitrogen in the bacteriological peptone.

An interesting pH variation (data not shown) during the cultivation of *H. salinarum* was observed that pH of all cultures increased with time before low oxygen condition was implemented. The pH increase was mainly resulted from the ammonium ions released by metabolizing amino acids for the cells growth. However, an apparent pH drop was observed after low oxygen condition was applied to enhance BR production. The pH drop may result from the increased amount of BR expressed because the existence of BR will actively pump proton from cytoplasm of cells to culture broth that lowers the pH of the culture. The pH of culture rose again at the end of cultivation is probably due to the amount of ammonium ions released from metabolizing amino acids was in excess of the amount of protons pumped out by BR.

Repeated-batch culture in bubble column photobioreactor

One of the reasons for the cells stop growing is due to the accumulation of some metabolites that inhibits the growth of cells. In order to enhance cell density achieved in the culture, the inhibitory metabolites need to be removed from the culture. The recycle bioreactor system equipped with an external membrane filter module has been employed for trying to grow *H. salinarum* in a higher concentration [14]. But this membrane bioreactor actually can only prevent the inhibitory metabolites from accumulating continuously as in a batch reactor. Especially, the cells in a high cell-density culture still encounter a high concentration of inhibitory metabolites. In order to reduce the effect of accumulated inhibitory metabolites on the growth of *H. salinarum* and BR production, repeated-batch with cell retention but medium replacement was employed to grow *H. salinarum* in shaker flask and bubble column photobioreactor using tryptone medium. The repeated-batch was operated by refilling the same amount of fresh tryptone medium into the cells pellet collected from previous batch culture by centrifugation.

As shown in Figure 4, the cell density of 4.8 g/L was achieved at 210 h after 3 and 5 repeated batches cultivation in the shaker flask and bubble column reactor, respectively. The timing for repeating the batch operation was every 48 h for bubble column reactor. In contrast, the medium replacement time in the shaker flask operation was at the moment when cells density did not increase for several hours. In

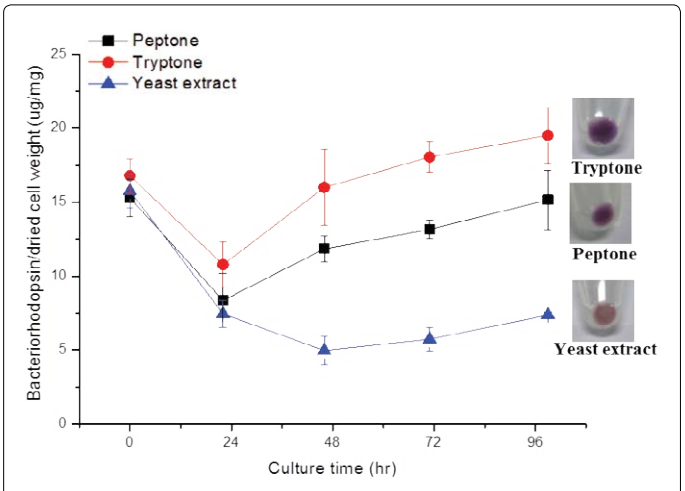

Figure 3: Bacteriorhodopsin contents in *H. salinarum* cultivated in bacteriological peptone, tryptone and yeast extract medium.

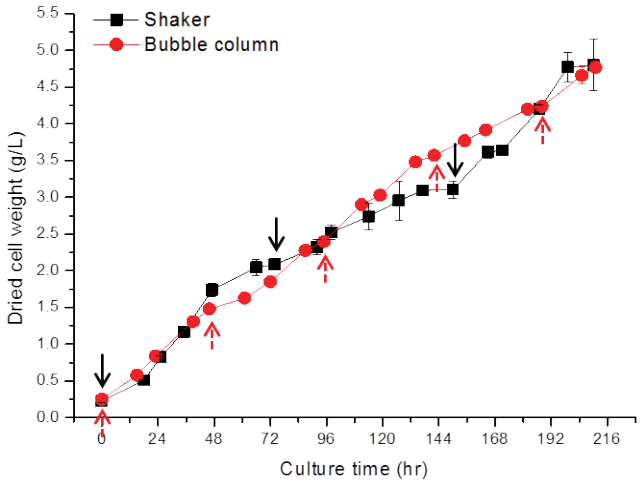

Figure 4: Repeated batch cultivation of *H. salinarum* in full-tryptone medium of a shaker flask and half-tryptone medium of a bubble column photobioreator, black-solid and red-broken arrow indicates full and half tryptone medium replacement.

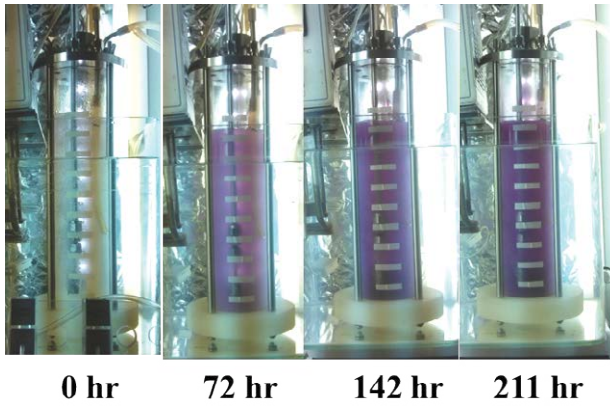

Figure 5: Images of *H. salinarum* cultivated with half-tryptone medium in a bubble column photobioreator under repeated batch operation.

addition, the tryptone concentration in the tryptone medium employed in bubble column was only half of that used in shaker flask. The photo images of the repeated batch operation of bubble column photo reactor are shown in Figure 5. The culture turned into purple color after 72 h and the color intensity increased with time. Apparently, the appearance of purple color in the culture is due to the presence of purple membrane in the cells. As shown in Figure 6, at the end of 210 h cultivation, BR concentration obtained in shaker flask and bubble column reactor is 148.8 mg/L and 201.8 mg/L, respectively. In terms of BR content per dried cell weight, 31 and 42.2 mg/g were obtained for shaker flask and bubble column reactor, respectively (Figure 7). The result indicates the half-tryptone concentration employed for the bubble column reactor not only satisfies the nutrients demanded for cell growth and BR synthesis but also reduces the level of inhibitory metabolites derived from tryptone. Providing half-tryptone concentration but increasing the frequency of medium replacement was not only able to keep cells grow exponentially that leads to an enhanced cell density but also reduce the medium cost for cell cultivation. The higher BR content in the cells obtained in bubble column reactor may be also due to the cells was exposed to a much better light illumination condition than in the shaker flask.

In shaker flask culture, the cells cannot synthesize more BR in cell membrane and remained steady BR content is probably because when cell density increased to certain level which will screen the light illumination for BR synthesis. In contrast, LED light was installed at the center of photobioreactor that can provide sufficient illumination and improves BR production effectively when the culture is grown to a high cell density.

Conclusion

Bacteriological peptone, tryptone, and yeast extract could be separately used in high salt concentration (25% NaCl) medium as carbon and nitrogen source for growing *H. salinarum* for BR production. Probably due to the presence of vitamin B complexes and

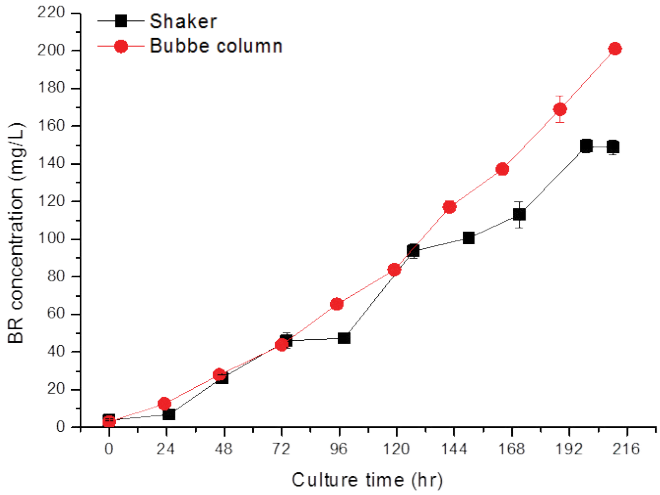

Figure 6: Bacteriorhodopsin produced by *H. salinarum* cultivated in full-tryptone medium of a shaker flask and half-tryptone medium in a bubble column photobioreator under repeated batch operation.

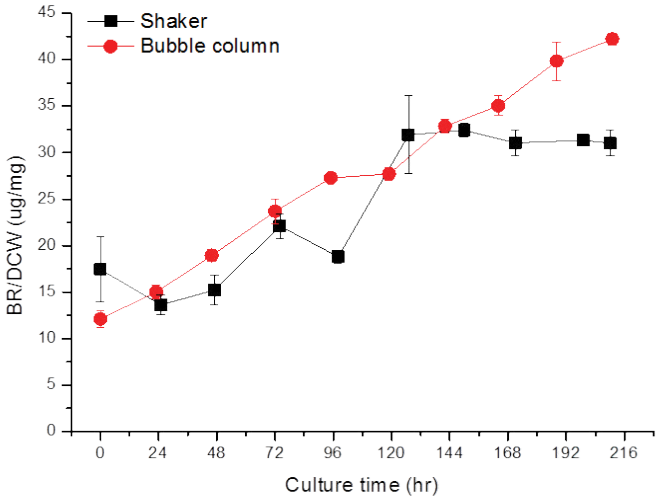

Figure 7: Bacteriorhodopsin content of *H. salinarum* cultivated in full-tryptone medium of a shaker flask and half-tryptone medium in a bubble column photobioreator under repeated batch operation.

growth factors, *H. salinarum* has a faster growth rate in yeast extract medium but with a lower BR production yield. Tryptone medium on the contrary resulted in a similar cell density as bacteriological peptone medium but about 1.4 fold higher for BR production. The repeated-batch operated in a bubble column photobioreactor with well illumination can grow *H. salinarum* into 4.8 g/L after 210 h with 5 repeated batches using half-tryptone concentration medium. The half-tryptone medium cultivation can reduce the medium cost into half but achieve similar cell density as in full-tryptone medium in a shaker flask. The present work demonstrated that using a well-illuminated bubble column photo reactor for repeated batch culturing *H. salinarum* is very beneficial for large-scale production of BR.

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