



Formulation and Viability of Consortia LiBeM Using Protective Agent Skim Milk Subjected to Freeze Drying Method for Degradation of Oil Sludge Contaminated Soil

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ABSTRACT

The formulation and viability of locally-isolated beneficial microorganisms (LiBeM) consortia was conducted by using protective agent skim milk at 30% (w/v). A 10% (v/v) of inoculum consists of mixed cultures *C. tropicalis*-RETL-Cr1+ *C. violaceum*-MAB-Cr1 + *P. aeruginosa*-BAS-Cr1 (RETL-Cr1+ MAB-Cr1+BAS-Cr1) were obtained from the Environmental Microbiology Laboratory, Universiti Malaysia Sabah and tested for biodegradation of oil sludge using the live cell powdered form. LiBeM formulation was carried out by using lyophilization under vacuum conditions for 24 hours. The viability of the initial cell loads on the final recovery after freeze drying process was measured weekly by heterotrophic plate counts (HPC). Surface morphological of LiBeM powder (LiBeM-POW) was observed under scanning electron microscopy (SEM) to show any changes on the surface cell structures. The results showed that the use of 10% (v/v) of inoculum with 10⁸ CFU/ mL as initial cell concentrations and 30% (w/v) skim milk as protective agent yielded 31.9% maximum viability. The findings also indicate that the survival rate of LiBeM cells have potential to survive and functionality even after 13 weeks of the storage with 10⁶ CFU/ mL recorded. The effectiveness of microbial formulation of LiBeM-POW was done by carrying out the biodegradation experiment in ASP-bioreactor system for 56 days. Two sets of ASP- bioreactor system containing 2 kg of soil mixed with 20% (v/v) of oil sludge were prepared as T1: treatment with consortia LiBeM-POW and T2: natural attenuation as a control plot. The results indicated that LiBeM-POW has potential to be applied in bioremediation of oil sludge contaminated environments with 92% degradation of TPH as compared to natural attenuation 26% degradation. This finding suggests the possibility of producing freeze dried powder of LiBeM consortia with high viability for prior used in bioremediation study. The comparison of other protective agent used by previous studied was also discussed in this paper.

INTRODUCTION

Biotechnology is the technology that involved living organisms that have been exploited widely especially in bioremediation studies. Preserving the microorganisms is an essential process in order to ensure their long term viability and genetic stability for prior used. According to a previous study [1] there are many preservation methods that can be applied to preserve bacteria such as freeze drying and cryopreservation beads. As for now, freeze-drying and ultra-freeze drying are considered as one of the most efficient methods to maintain cells viable and genetic consistency [2]. Freeze drying method or known as lyophilisation or cryodesiccation is a low temperature dehydration process which involved freezing the bacterial cell, lowering pressure thus

removing the ice by sublimation. This method sufficiently active to produce rapid growth and it can be held a long time without losing its activity.

In this context, the inoculants carriers have been used to improve effectiveness by supplying nutrients, protection from desiccation and slow cell release. In completely, dry formulation, bacteria remain in a dormant form, its metabolism is very slow or even stopped and are resistant to environmental stress, insensitive to contamination and are more compatible with various application. However, these techniques require a specialized and expensive equipment and sometimes may cause severe damages to bacteria cell wall, cell membranes, DNA and proteins [1,3].

This is due to the side effect and intermediates process of recovery the viable cell is very crucial [4].

To improve this, researchers have work on preservation process and parameters involved in order to develop a new technique that can produce high viability of cells for a long term used. The addition of various protective agent in the microbial formulation have been introduced widely in order to protect the cell during the freeze-drying process and to ensure the desiccations tolerance of microorganisms. Protective agent such as skim milk, calcium carbonate, sucrose, fructose, mannitol and microencapsulation are known to protect the microbial cell wall, provide pH stabilization, increase the survival rates of microbial cells and increase the protection against extreme process conditions [5-7]. Furthermore, these protective agents can stabilize the proteins and membranes of bacterial cell by replacing the water with sucrose and trehalose [8]. The main objective of this study is to evaluate the formulation and viability of locally isolated beneficial microorganism (LIBeM) consortia using specific protective agent skim milk subjected to freeze drying method. The effectiveness of microbial formulation LIBeM powder was tested in bioremediation study of oil sludge contaminated soil. The biodegradation efficiency, the degradation of n-alkanes and polycyclic aromatic hydrocarbon (PAHs) compounds were also discussed in this paper.

METHODS

Sources of Microorganisms

The unique strains of LIBeM were isolated from two different places; Titan Petrochemical (M) Sdn. Bhd (TPSB) Pasir Gudang Johor and Exxon Mobil Oil Refinery (EMOR) Port Dickson Negeri Sembilan [9,10]. These microorganisms have been proven to degrade oil and phenol based on the previous research done by [9,10].

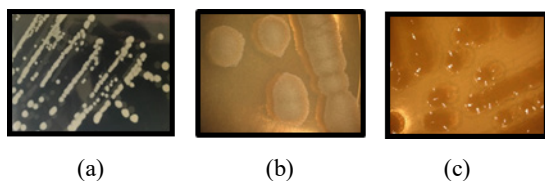


Fig 1. Selected strains of LIBeM (a) *C. tropicalis* –RETL-Cr1, (b) *C. violaceum*-MAB-Cr1 and (c) *P. aeruginosa* obtained from Environmental Microbiology Laboratory, FSSA, Universiti Malaysia Sabah (UMS).

Inoculum Preparation of LIBeM

LIBeM consortium consists of *Candida tropicalis*-RETL-Cr1+ *Chromobacterium violaceum*-MAB-Cr1 + *Pseudomonas aeruginosa*-BAS-Cr1 were cultured to 1.5 L Ramsay broth in 2 liters Erlenmeyer flasks with 2 mM of MgSO₄.7H₂O and 5 mM of glucose were added as a nutrient medium. The flasks were incubated in an orbital shaker agitated at 200 rpm for 30 °C within 24 h. After incubation, the OD was assessed by using spectrophotometer (Model CECIL CE 1011) set at 600 nm for preparation of microbial formulation.

Protective Agent of LIBeM

The protective agents used in this study were skim milk brand (R & M Chemicals) as a source of Ca²⁺. It contains 1.5% of milk fat with natural milk powder and highly nutritious for microbiological used.

For microbial formulation of powder, 30% freshly skim milk were dissolved in 1 liter of sterile distilled water as requirement of each experiment. The selection of this protective agent was based on a preliminary study [11] with some modifications.

Preparation of Microbial Formulation LIBeM Powder for Freeze Drying

The microbial formulations of LIBeM powder were done by harvesting the cells at the early stage of stationary phase under aseptic condition by centrifugation for 10 min at 12,857x g (Eppendorf, Centrifuge 5810R). Harvested cells about 100 mL were washed twice using 0.02 M phosphate buffer at pH 7.2 [11]. The cell pellets were then re-suspended in the same volume of skim milk (30%) as protective agent. Each suspension (20 mL) was transferred into a sterile universal bottle and chilled at 4°C for 2 h [11]. The chilled suspension was frozen under freezing condition at -80°C for 24 hours.

The effectiveness of microbial formulation using protective agents to maintain viability of microbial strain LIBeM in powder form was done using lyophilization or freeze method drying under vacuum condition (freeze dryer 6L Model LABCONCO). This stabilizing process in which a solution of substance is first frozen and then quantity of the solvent is reduced first by sublimation and then by desorption to a value that will no longer support biological activity or chemical reaction. The protective agent skimmed milk was selected in this experiment as operating variables and survival of cultures after freeze drying process.

Determination Viability of LIBeM

Methods for the determination viability of LIBeM powder depend on examination of a variety of cell characteristics [12]. However, in this study heterotrophic plate counts (HPC) were carried out immediately after freeze drying using APHA standard method 9215. The viability of LIBeM was measured by calculate the % of survival rate as mentioned in the formula below [11].

$$\% \text{ of survival} = \frac{\text{number of survival cell}}{\text{original number of cell}} \times 100\%$$

Scanning Electron Microscopy (SEM) Observation

The surface morphology of LIBeM were examined by scanning electron microscopy. The SEM sample preparation of powder LIBeM were done by three steps; 1) sample cleaning; 2) sample undergone freeze drying method and 3) sample coating [13]. The dried samples were mounted on the copper stubs using adhesive carbon tapes. Gold was sputtered on the material to form 20 nm thick layers on the surface. The SEM images were captured at 15 kV accelerating Voltage using SEM (Model ZEUS).

Experimental Set-up for Biodegradation of Oil Sludge by LIBeM-POW

The effectiveness of microbial formulation LIBeM-POW was tested by carrying out the biodegradation experiment in an ASP-bioreactor system with dimension of 60 cm x 40 cm x 20 cm (**Fig. 2**). The experiments (duplicates) for each ASP-bioreactor were carried out for 56 d at open and air ventilated with following treatment combination:

Table 1. Biodegradation treatment of high concentration of oil sludge (20% v/v) by LIBeM-POW.

Treatment Plot	Inoculation
LIBeM-POW	2 kg of soil were treated with LIBeM-POW; <i>C. Tropicalis</i> + <i>C. violaceum</i> + <i>P. aeruginosa</i> (Retl-Cr1 + Mab-Cr1 + Bas-Cr1)
NA	2 kg of soil were treated with indigenous microorganisms in soil. Non inoculation of bacteria into the soil (control plot)

**Fig 2.** Biodegradation of oil sludge contaminated soil by (a) LIBeM-POW and (b) NA within 56 days.

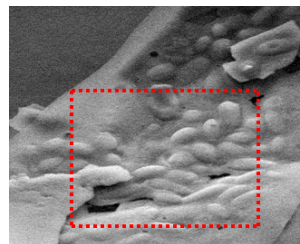
RESULTS AND DISCUSSION

Table 2 showed the effect of skim milk protective agent on the survival rates of LIBeM after freeze drying process. The results demonstrated that the cells of LIBeM which were freeze-dried with skim milk as individual protective agent had substantially higher survival rates with 31.9%. It is important to note that as the % of survival rate increased, it indicates that the viability of cells is in maximum conditions for further used. The data explained that after freeze drying process, the survival rate of LIBeM recorded is 2.9×10^8 CFU/mL from 9.1×10^8 CFU/mL. However, along the periods of time (week 16), the survival rate of LIBeM is found to decrease to 6.0×10^6 CFU/mL which is 48.3-fold lower than week 1. This is due to the changes of the physical state of membrane lipids that may result in severe loss of bacterial viability during storage [14].

Table 2. Effect of Protective Agent Skim Milk on Survival Rate of LIBeM after freeze drying process.

Time (Weeks)	Enumeration of LIBeM	
	Viable Cell (CFU/mL)	Survival Rate (%)
Before Freeze Drying	9.1×10^8	-
After Freeze Drying		
Week 1	2.9×10^8	31.9
Week 2	1.3×10^8	14.3
Week 3	9.5×10^7	10.4
Week 4	4.5×10^7	4.94
Week 5	4.4×10^7	4.83
Week 6	4.3×10^7	4.72
Week 7	3.9×10^7	4.28
Week 8	2.5×10^7	2.74
Week 9	2.5×10^7	2.74
Week 10	2.3×10^7	2.52
Week 11	2.2×10^7	2.41
Week 12	1.9×10^7	2.08
Week 13	1.1×10^7	1.20
Week 14	9.0×10^6	0.98
Week 15	8.5×10^6	0.93
Week 16	6.0×10^6	0.66

The results also showed that after 16 weeks of storage, the survival rate of LIBeM is drop to 0.66% (6.0×10^6 CFU/mL). **Fig. 3** showed the surface morphology of consortium *C. tropicalis*-RETL-Cr1+*C. violaceum*-MAB-Cr1+ *P. aeruginosa*-BAS-Cr1 under scanning electron microscopy (SEM) after the freeze-drying process.

**Fig 3.** Scanning electron microscopy (SEM) images of surface LIBeM consortium at 1.5k magnification. Area enclosed by dotted line is the field of view detectable specimen.

This finding indicates that formulations of LIBeM in powder form have potential to survive and functionality even after 16 weeks of storage. This is information is very useful for application of LIBeM for further used in bioremediation studies. Survival of microorganism during formulation is affected by several variables: the culture medium used for bacteria growth, the physiological state of the bacteria when harvested from the medium, the process of cell encapsulation, the use of protective materials, the type of drying technology, and the rate of dehydration. If properly dehydrated, the shelf life of the dried formulation is much longer than any moist product [15].

According to the data obtained, it can be conclude that skim milk (30% w/v) used in this study provide the highest degree of protection compared to other protective agent reported by Lim *et al.*, (2009), which exhibited only 9%, 0.08% and 1.3×10^{-5} CFU/mL when using sucrose, glycerol and calcium carbonate (Ca^{2+}). Skim milk is the most useful protective agent for freeze drying because of its good cryo-protective effect as mentioned previously [16].

Additionally, milk proteins may form a proactive coating on the cell wall of LIBeM, and calcium in the milk increase the survival rate after freezing of freeze-dried cells [6]. Carvalho *et al.* [17] reported that the role of protective agent skim milk as drying medium can stabilize the cell membranes constituents by creating a protective coating over the cells. It also optimized bacteria survival and reduces membrane damage during and after freeze drying process. Modler *et al.* [5] also reported that skim milk which contains many solutes such as phosphates and citrate that would provide buffering capacity for pH stabilization for protective cells. This finding is in line with [18] who reported that strains *Candida sake* cell recoveries ranged from 45-85% using a mixture of skimmed milk and carbohydrates sugars. Similar results have been found by [19] asserted that survival of *Lactobacillus puracasei* was significantly increased up to 100-fold when 10% gum acacia was added to 10% reconstituted skim milk. It would therefore appear that the most efficient desiccation protective agents are a mixture of proteins and sugar as aforementioned. **Table 3** showed the comparable percentage of survival rate used by previous study with various protective agents after freeze drying process.

Table 3. Comparative Effects of various protective agents on the survival rates of bacterial after freeze drying process.

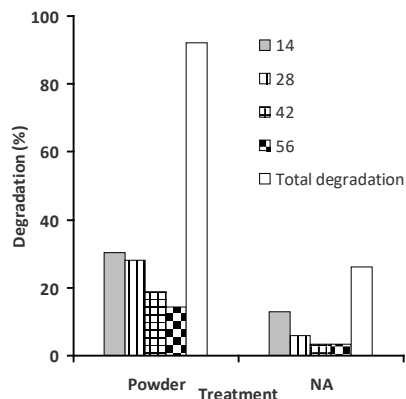
Strains	Protective agent	Viable cells (CFU/mL)		% of survival	Ref
		Before freeze drying	After freeze drying		
	distilled water	1.3×10^{10}	1.0×10^7	0.08	
	skim milk (20% w/v)	1.1×10^{10}	1.4×10^9	13.03	
<i>L. salivarius</i>	sucrose (20% w/v)	5.0×10^9	4.5×10^8	9	[11]
	glycerol (5% v/v)	5.4×10^9	7.1×10^2	1.3×10^{-5}	
	calcium carbonate	4.1×10^{10}	Below detection limit	0.00	
	sucrose (10% w/v)	NS	NS	45.1	
<i>L. brevis</i>	maltose (10% w/v)	NS	NS	41.2	
	glucose (10% w/v)	NS	NS	18.6	[21]
	fructose (10% w/v)	NS	NS	14.2	
	sorbitol (5% w/v)	NS	NS	10.6	
	mannitol (5% w/v)	NS	NS	11.8	
	<i>C. tropicalis</i>	Skim milk (30% w/v)	9.1×10^8	2.9×10^8	31.09
RETl- <i>C. violaceum</i> -MAB- <i>C. aeruginosa</i>					

*NS=Not stated

As shown in **Table 3**, the used of protective agent skim milk in this study has accelerated the maximum survival rate of LIBeM with 31.9%. There are a number of cryoprotective agents such as proteins, sugars and carbohydrates have been used to minimize the bacterial inactivation after freeze drying and subsequent storage [17]. Most studied have reported that skim milk and sucrose were better protective agent than glycerol and calcium carbonate [11,18-20]. However, Zhou et al. [21] found that the use of sucrose (10% w/v) and maltose (10%w/v) have accelerated the survival rate up to 41.2% and 45.1% which 1.4-fold higher than present work.

Biodegradation of Oil Sludge Contaminated Soil by Microbial Formulation LIBeM-POW

The biodegradation of oil sludge contaminated soil throughout the period of 56 days are shown in **Fig. 4**. The results showed that high percentage degradation of TPH was observed in soil treated with LIBeM-POW as compared to natural attenuation. At the end of the incubation days, the TPH was reduced from 200 g/kg to 16.7 g/kg and 148.9 g/kg by soil treated with LIBeM-POW and NA respectively. This represents TPH biodegradation efficiency of 92% and 26% in both treatment sets. Treatments using microbial formulation of LIBeM-POW have greater oil biodegradability with 2.4-fold higher as compared to NA. As shown in **Fig. 4** the highest biodegradation efficiency in LIBeM-POW mostly occur within 14-28 days incubation with 68.5% degradation.

**Fig 4.** Biodegradation efficiency of LIBeM-POW and natural attenuation (NA) treatment during 56-day bioaugmentation period.

When inoculations of LIBeM-POW take place in contact with oil sludge, they immediately start to lock up and contain the spilled materials. In the presence of moisture content in soil, LIBeM-POW was activated and start to break down and digesting the oil. The strong binding between LIBeM-POW with oil sludge help LIBeM-POW to speed up the rate of biodegradation. These strains start to utilize and consume oil sludge into nontoxic byproduct such as carbon, carbon dioxide and lipids. By the end, these byproducts are used again as a source of energy to multiply. LIBeM-POW also is known to well adapt for land, water and hard surface. This microbial formulation is easy to use and handle with non-toxic, non-corrosive and is biodegradable.

In context of bioremediation of oil sludge, the successful of bioremediation is mainly focused on the ability and efficiency of the strains used to degrade high percentage of oil subjected to short times taken. Therefore, treatment using LIBeM-POW is the most utter strategies in order to achieve the great removal of TPH in soil with a short time needed.

Degradation of Aliphatic Hydrocarbon (n-alkanes) and aromatic hydrocarbon (PAHs) by LIBeM-POW

The GC-MS analysis of oil sludge detected heptadecane ($C_{17}H_{36}$), octadecane ($C_{18}H_{38}$), nonadecane ($C_{19}H_{40}$), eicosane ($C_{20}H_{42}$), heneicosane ($C_{21}H_{44}$), docosane ($C_{22}H_{46}$), tricosane ($C_{23}H_{48}$) and tetracosane ($C_{24}H_{50}$) is shown in (**Fig. 5a**). The results reveal that this fraction of oil sludge was properly degraded more than >80% in all treatment LIBeM-POW compared to natural attenuation. It was found that the long chain alkanes except tricosane were degraded to 83%, while some of these compounds were completely degraded after 56 days of incubations. The preference degradation compound of LIBeM-POW is docosane (99.2%)> tetracosane (99.2%)>Eicosane (99.1%)> heneicosane (99.0%)>octadecane (98.8%)> nonadecane (98.7%) > heptadecane (97.6%)> tricosane (88.3%). This marked of 3.3-fold higher as compared to NA.

According to Tebyanian et al. [22], many organisms were found to grow on or can easily metabolically oxidize alkanes. In other similar study, the degradation of long chain alkane's hydrocarbons was observed higher in bioaugmentation of mixed *Acremonium* Sp. and *B. subtilis* which significantly promoted degradation for all hydrocarbon fraction with >95% respectively. Xiao et al. [23] reported that treatment by mixed consortia above was much better than indigenous strains solely in the soil. Our findings supported that treatment by LIBeM-POW which consists of consortia strains (*C. tropicalis*-RETL-Cr1 + *C. violaceum*-MAB-Cr1 + *P. aeruginosa*-BAS-Cr1) have greater degradation capability in degrading oil sludge contaminated soil.

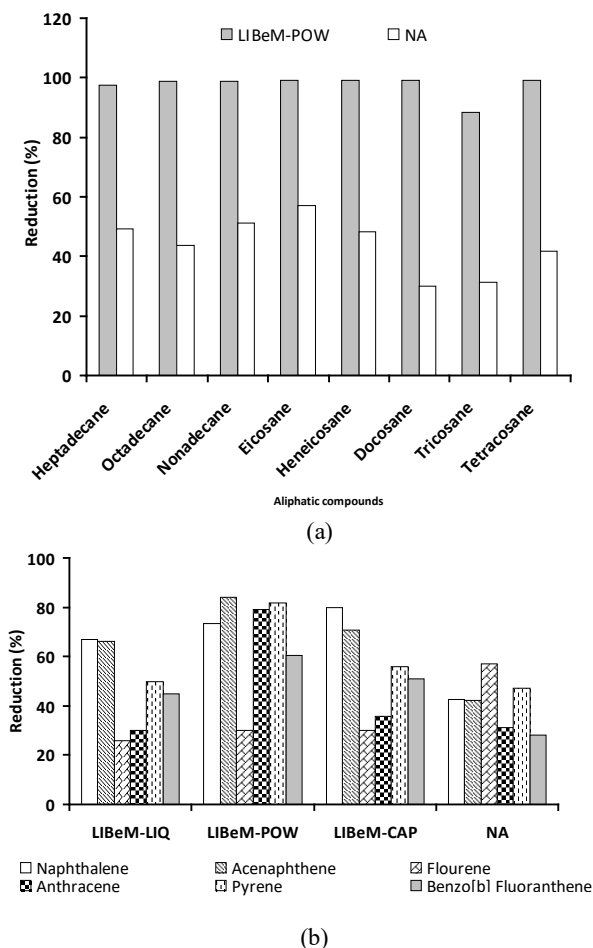


Fig 5. Degradation of (a) aliphatic hydrocarbons (n-alkane) and (b) aromatic hydrocarbon (PAHs) by LIBeM-POW and NA during 56-day treatment period.

The degradation of polyaromatic hydrocarbons compound composed of 2, 3, 4 and 5 benzene rings namely naphthalene ($C_{10}H_8$), acenaphthalene ($C_{12}H_8$), flourene ($C_{13}H_{10}$), anthracene ($C_{14}H_{10}$), pyrene ($C_{16}H_{10}$), and benzo [b] flouranthene ($C_{20}H_{12}$) is shown in **Fig. 5b**. Based on the results obtained, oil sludge contaminated soil treated with LIBeM-POW recorded highest degradation of six PAHs compound ($C_{10} - C_{20}$) with 30%- 82% as compared to natural attenuation plot. Empirically, the first sign of biodegradation is usually occurred in simplest PAHs compounds which are naphthalene and acenaphthalene fraction. However, in this present work, the rapid degradation by LIBeM-POW can be observed in pyrene with 82%.

It was reported that bacteria that capable of degrading the larger PAHs compounds are often be able to degrade the smaller ones and the reverse is not generally true [24]. In fact, the PAHs degradation in soil is most effective when there is a broad diversity of degrading microorganisms added. It was clearly showed that the selected of PAHs compound degraded by LIBeM-POW in ASP-bioreactor systems are in sequence as follow; Pyrene > Acenaphthene > Anthracene > Naphthalene > Benzo[b]Fluoranthene > Flourene. Natural attenuation treatment had the least biodegradation of PAHs hydrocarbon fraction (28% - 57%) throughout 56 days periods. These findings proved that indigenous microorganism in soil were able to degrade the small percentage of PAHs compound in the soil. A previous study [25] reported that some species are capable in degrading aliphatic hydrocarbons and aromatic hydrocarbons, but few are able to degrade both classes of these molecules.

In this present work, LIBeM-POW are proved to degraded both fraction molecules, but the efficacy of hydrocarbon degrader are more subjected to aliphatic compounds. This finding suggest that our LIBeM-POW consist of community of strains that able to sequentially exchange and then transform excreted metabolites molecules to be broken down [26]. Considering the above results mentioned, the biodegradation of PAHs by LIBeM-POW in the experiment could not be omitted. It can be concluded that the quick decomposition of 2 benzene rings of PAHs (naphthalene and acenaphthalene) occur rapidly after the final day of experiment. Hence it can be presumed that the groups of LIBeM-POW added were capable in biodegradation of oil sludge introduced to the soil.

CONCLUSION

In this study, lyophilization method has been used as dehydration process for LIBeM consortia in order to achieve a solid formation. Skim milk at 30% (w/v) has been selected as protective agent for freeze drying and resulting in high degree survival rate of LIBeM with 2.9×10^8 CFU/mL (31.9%). The biodegradation study of microbial formulation LIBeM-POW has showed a great performance in degrading TPH with 92% as compared to NA. This can be proved with high degradation of aliphatic and aromatic compounds after 56 days treatment. It can be concluded that the developed technology on microbial formulation of LIBeM-POW can be used as utter strategies in order to achieve the great removal of TPH degradation in soil with a short time needed.

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