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# Effect of Fermentation Period on Itaconic Acid Production from Plantain peel using *Aspergillus niger* and *Aspergillus flavus* in Solid State Fermentation

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## ABSTRACT

Itaconic acid (IA) is a highly valued product in the industry and plantain peel is a promising substrate for its production. This study evaluates the peel of plantain for production of Itaconic acid through solid-state fermentation using resident fungi. Plantain peel was collected; proximate analysis and fermentation were done using standard methods. Fungi were isolated and identified using standard microbiological procedures. Fungal screening for IA production was conducted using Czapek-dox agar with Bromocresol purple as an indicator. pH, total titratable acidity (TTA), reducing sugar, fermentation period was achieved by standard methods. Itaconic acid was quantified colorimetrically at 385 nm. Proximate composition revealed carbohydrate content (54.44%), lipid (4.99%), fiber (7.92%), ash (15.93%), moisture (7.80%), and protein (8.92%). Fungal isolates were identified as *Aspergillus niger* and *Aspergillus flavus* and showed positive reaction for production of IA. TTA values ranged from 15.76 mg/mL on day 1 to 6.66 mg/mL on day 2, with pH fluctuating between 3.8 (day 5) and 2.0 (day 1). Reducing sugar peaked on day 2 (3.26 mg/mL) and reduced to 0.38 mg/mL by day 7. Maximum Itaconic acid production (9.43 ± 0.02 mg/mL) occurred on day 2, while the lowest Itaconic acid was obtained on day 5 (1.25 ± 0.01 mg/mL). The findings demonstrate that *Aspergillus flavus* and *Aspergillus niger* are effective for Itaconic acid production from plantain peel under optimized conditions.

**Keywords:** Plantain peel, Itaconic acid fermentation, *Aspergillus niger*, *Aspergillus flavus,* Solid-state fermentation.

## INTRODUCTION

Fermentation is an anaerobic process where microorganisms change organic matter, for examples sugars into less complex products like alcohol, gases and acids. It produces power in contexts of low or lack of oxygen – this explains how cells can endure (Madigan *et al*., 2020). Fermentation can be achieved by bacteria, yeast and filamentous fungi in which various pathways are utilized by a fermentation type, species and the surroundings that they undergo, and they include lactic acid, alcoholic and mixed-acid fermentation (Stanbury *et al*., 2016).

These organisms are important in many industries, for instance food processing industries, wine and brewing industries as well as pharmaceutical industries and newly developing bio-energy industries that use fermentation processes to produce goods like bread, yoghourt, beer, ethanol and antibiotics among others. In addition, some fungal strains such as. *A. niger* and *A. flavus* are used in synthesizing organic acids They are chiefly employed to produce itaconic acid, which is an important organic acid employed in the synthesis of biodegradable polymers, adhesives, and resins (Weusthuis *et al*., 2021). The suitability of these fungi to metabolize a rich carbohydrate substrate such as the complex lignocellulosic biomass or simple sugars for the synthesis of itaconic acid is now becoming a basic tool in biotechnological processes.

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Itaconic acid, a valuable organic compound, has gained significant attention due to its versatility in industrial applications, including the production of bio-based polymers, resins, and as a precursor for pharmaceuticals. The increasing demand for sustainable and eco-friendly production methods has driven research into alternative sources and production techniques for this acid. Traditional production methods rely on petrochemical processes, which are unsustainable and contribute to environmental degradation. Consequently, there is rapid interest in biological production methods, particularly through microbial fermentation to convert waste into valuable industrial products (Siddiqui *et al.,* 2023).

Fermentation by solid-state techniques includes an increase in size and number of microbes on a solid mass having less or no free water. Also, it involves the utilization of different substrates, comparatively cheaper and environmentally friendly than submerged fermentation (SF) (Rodrigues *et al*., 2020). It has emerged as an advantageous method for producing organic acids, including itaconic acid, due to its cost-effectiveness and minimal water requirement. SSF mimics natural environments where microorganisms thrive on solid substrates, making it particularly suitable for agricultural waste valorization (Okafor *et al.,* 2022). Among the various substrates explored for SSF, ripe plantain peel (*Musa paradisiaca*) presents a promising option. Plantain peel is an abundant agro-waste in tropical regions, and its rich carbohydrate content makes it an ideal candidate for fermentation processes (Adebayo *et al.,* 2023).

Plantain peel is an agricultural waste which is discarded thus polluting the environment due to their high volume in weight resulting in substantial waste generation, therefore its proper utilization will enhance reduction in environmental pollutions. However, there is a strong relationship between the fermentation period and the yield and efficiency of the fermented plantain peels (Fischer *et al*., 2019). This research was aimed at analyzing the effects of fermentation period on the yield of itaconic acid from naturally occurring fungi under solid state technique conditions.

## MATERIALS AND METHODS

### Sample Collection

Fresh plantain peels were sourced from Chop Life Restaurant in Kwara State University, Malete and brought to the Department of Microbiology Laboratory. The peels were rinsed with distilled water to get rid of debris before they were oven dried at 40 $^{o}$C for 90 minutes when they turned crispy (plate I). After cooling to room temperature (28°C), the dried peels were pulverized using a Twister Mixer Grinder (Model: 410025). The powdered substrate was also kept in an airtight jar where it would be used again. Each step was performed under aseptic technique to avoid microbial contamination (Maliki *et al*., 2023).

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Plate 1: Ripe Plantain Peels

**Preparation of Culture Media**

The media employed in this study were Czapek Dox Agar and Potato Dextrose Agar (PDA). Both were made as specified by the manufacturer's protocol. The manufacturers' instruction was strictly followed in preparation of Potato Dextrose Agar. The medium was sterilized at 121°C for 15 minutes in the autoclave and allowed to cool before usage. Czapek Dox Agar was prepared in the same way following the manufacturer's instruction for the texture and appearance as described above. The medium was autoclaved and maintained at room temperature.

**Physicochemical Analysis of Plantain Peels**

The following physiochemical properties of the plantain peel substrate were determined: Proximate composition analysis include moisture content according to AOAC (2019), ash, crude protein, crude fat, crude fiber and quantitative carbohydrate content. pH was measured using a pH meter (HANNA pH meter, Model H196107) while titratable acidity was determined through titration with NaOH (AOAC, 2019).

**Isolation of Resident Fungi from Plantain Peel**

Plantain peel (20 grams) was dispensed in a clean container and mixed with 50 mL of sterile distilled water for natural fermentation to occur for 7 days at room temperature (Kayode *et al.,* 2008; Ajiboye *et al.,* 2018). Thereafter, the fermented substrate (1 g) was diluted with 9 mL of sterile distilled water in test tubes and dilutions up to $10^{-7}$ were done.

Using the spread plate method, an aliquot (0.1 mL) from each test tube was placed on PDA which has been incorporated with streptomycin (0.1 mL) and incubated for 3 days at 28°C (Fawole and Oso, 2004). Fungal isolates were sub-cultured repeatedly to obtain pure cultures. They were then characterized and identified macroscopically and microscopically according to Duncan (2017), after lactophenol cotton blue staining as described by Fawole and Oso (2004) was carried out.

**Screening of Fungal Isolates for Itaconic Acid Production**

Resident fungi were qualitatively screened for production of itaconic acid by plate method

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using Czapek-dox agar containing Bromocresol purple as indicator. The spore suspension of the isolates was put in the agar wells of 5 mm in diameter of the medium plates and allowed to grow for 7 days. Zones of yellow coloration indicated itaconic acid production (Sindhu *et al.,* 2023).

**Preparation of Inoculum for Fermentation**

Preparation of spore suspensions of the fungal isolates was done by adding sterilized distilled water (10 mL) to the sporulated seven (7) days old culture. A sterile inoculating needle was used to disengage the spores, and it was mixed thoroughly to prepare a uniform spore suspension under aseptic conditions (Grigoryev, 2013; Ajiboye *et al.,* 2023).

**Determination of pH from Fermented Plantain Peel by the Fungal Isolates**

The pH of fermented plantain peel by co-cultures of *Aspergillus flavus* and *Aspergillus niger* were monitored on each day of fermentation using pH meter (Model H196107).

**Total Titratable Acidity (TTA) Determination**

The total titratable acidity was obtained by titration of the culture filtrates against standard 0.1N NaOH solution (Ahmed *et al.,*2015). The indicator used was phenolphthalein.

**Determination of Reducing Sugar**

Dinitrosalicylic acid (DNSA) method was used to estimate the reducing sugar content. This was determined by taking a 1.0 mL of the diluted solution (1 mL sample in 100 mL distilled water) with 3.0 mL of DNSA reagent in a test tube. The tubes were heated in a boiling water bath for approximately 5 minutes. After cooling the tubes at room temperature, 40% Rochelle salt solution (1 mL) was added to the tube and absorbance was read at 510 nm using a spectrophotometer. Standard curve of glucose was used to determine the sugar concentrations (Miller, 1959; NCBE, 2016).

**Solid State Fermentation Process for Itaconic Acid Production**

This was carried out by weighing 5g of plantain peel substrate into 7 bottles each and dissolved in 10 mL sterile distilled water to which 15 mL of the nutrient solution of the following composition $(NH_4)2SO_4$ (2.36 g/L), NaCl (0.074 mg/L), $FeSO_4.7H_2O$ (0.01 mg/L), Glucose (40.0. g/L), $KH_2PO_4$ (1.0 g/L), $MgSO_4$ (0.5 g/L), $CaCl_2$, (0.13 g/L), $CuSO_4.5H_2O$ (0.2 mg/L), and $ZnSO_4.7H_2O$ (1.3 mg/L) in distilled water (Mokula *et al.,* 2014; Blaga *et al.,* 2018) were added. The contents were autoclaved at 121ºC for 15 mins.

The spore suspension containing $1x10^7$ spores/ mL of *Aspergillus niger* and *Aspergillus flavus* were added as inoculum in solid state fermentation process after sterilization (Ajiboye *et al.,* 2023). The effect of different incubation periods (1 – 7 days) was determined. After the fermentation, the fermented substrate (1 gram), was added to 50 mL of distilled water, it was thoroughly mixed and made to stand for 30 mins before it was filtered. The filtrate was used for the determination of itaconic acid concentration, reducing sugar, pH and titratable acidity. The Itaconic acid production by the *Aspergillus* species was studied daily for 7 days. The experiments were conducted in replicates and the mean values were documented.

**Extraction and Assay of Itaconic Acid**

The filtrate obtained from the fermented substrate was utilized as crude itaconic acid. The itaconic acid content was determined by colorimetric method (385 nm). A standard curve for itaconic acid was used to quantitate itaconic acid produced (Mokula *et al.,* 2014). To assay for itaconic acid, 0.3 mL bromine

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reagent was dispensed in 3 mL cuvette and was made up to 1.0 mL with distilled water. Hydrochloric acid (HCL) of pH 1.2 was added to make up to 3.0 mL and allowed to stand for 15 mins (this served as the blank). Into a different 3 mL Beckman cuvette, 0.3 mL bromine reagent, sample (1.0 mL) and HCl (pH 1.2) were added to make it 3.0 mL volume. The absorbance was read at wavelength 385 nm after 15 mins.

### Itaconic Acid Quantification

Itaconic acid concentration in the extract was obtained using the standard curve for itaconic acid. The absorbance readings of the fermentation samples were read on the standard curve to estimate itaconic acid concentration as described by Lemoine *et al*. (2018).

### Statistical Analysis

Statistical analysis of the data was processed to estimate the mean ± standard deviation (SD) and one-way analysis of variance (ANOVA). The statistical analysis was done with the Statistical Package for the Social Sciences (SPSS) version 16.0 (SPSS Inc., Chicago Illinios, USA), the level of significance used in the study was $p < 0.05$.

## RESULTS

### Proximate Composition of Plantain Peels

The proximate composition analysis on plantain peel brought moisture content to 7.80% with no variation, then protein content (8.92±0.40 %) showed slight variation. The average ash content was 15.93±0.15 %, which suggested moderate level of inter-batch Coefficient of Variation and the crude fiber content was 7.92±0.40 %. It had a fat content of 5.00±0.15 % and the major component was carbohydrates that comprised of 54.44±0.50 %. The findings presented here show that peels have nutritional values with regard to its composition being mainly carbohydrates, ash, protein, fiber and fat. This is concluded in Table 1.

**Table 1:** Proximate Composition of Plantain Peel.

| Parameters | Value (%) |
|---|---|
| Protein Content | 8.92 ± 0.40 |
| Moisture Content | 7.80 ± 0.00 |
| Ash Content | 15.93 ± 0.15 |
| Crude Fiber Content | 7.92 ± 0.40 |
| Fat Content | 5.00 ± 0.15 |
| Carbohydrates | 54.44 ± 0.50 |

Values are means of duplicate reading and SEM of proximate composition of plantain peel

### Physicochemical Composition of Plantain Peel before Fermentation

The Physicochemical composition of the plantain peel obtained was pH (5.35 ± 0.05) and titratable acidity (53.0 ± 3.0 mL) as shown in Table 2.

**Table 2:** Physiochemical Composition of Plantain Peel.

| Physicochemical Parameters | Physicochemical Value |
|---|---|
| pH | 5.30 ± 0.05 |
| TTA (mg/mL) | 53.0 ± 3.00 |

Values are means of duplicate readings and SEM of physicochemical composition of plantain peel

### Characterization of Fungal Isolates from Plantain Peel

Two fungal isolates were obtained from the fermented plantain peel: In this study, F1 with the characteristic of black spots, white mycelium, large and depressed colony morphology was confirmed to be *Aspergillus niger*. The second isolate F2 characterized by white mycelia, greenish spores, accompanied by large, flat growth was identified as *Aspergillus flavus*. A summary of these findings is presented in Table 3.

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**Table 3:** Characterization of Fungal Isolates from Fermented Plantain Peel.

| Fungal Isolates | Macroscopic and Microscopic Description | Probable Organisms |
|---|---|---|
| F1 | Black spores on the top and whitish at the reverse, large, it has a septate hypha bearing conidiophores which are head long and slender. | *Aspergillus niger* |
| F2 | Yellowish green spores, whitish mycelia with septate hyphae bearing conidiophores, which are rough and colorless. | *Aspergillus flavus* |

## Screening Reaction of Fungal Isolates

The ability of the fungal isolates to show zones of yellow coloration were observed with the two *Aspergillus* spp showing positive reactions as further shown in Table 4.

**Table 4:** Screening Reaction of Fungal Isolates for Production of Itaconic Acid.

| Fingal Isolates | Screening Reaction |
|---|---|
| *Aspergillus niger* | Positive (+) |
| *Aspergillus flavus* | Positive (+) |

## pH of Fermented Plantain Peels for Itaconic Acid Production by *Aspergillus flavus* and *Aspergillus niger*

The highest pH value (3.8) of fermented plantain peel was obtained on Day 5 while the lowest pH value (2.0) was obtained on Day 1 (Table 5).

**Table 5:** pH of Fermented Plantain Peel by *Aspergillus niger* and *Aspergillus flavus* at Different Fermentation Periods.

| Fermentation Period (day) | pH |
|---|---|
| 1 | 2.0 |
| 2 | 3.0 |
| 3 | 2.3 |
| 4 | 2.6 |
| 5 | 3.8 |
| 6 | 3.5 |
| 7 | 3.0 |

## Total Titratable Acidity of Fermented Plantain Peel by *Aspergillus niger* and *Aspergillus flavus.*

The highest TTA concentration was obtained on day 1 (15.76 ±1.88 mg/mL) and the lowest concentration of TTA was obtained on day 2 (6.66±1.48 mg/mL) (Table 6).

**Table 6:** Total Titratable Acidity of Fermented Plantain Peel by both *Aspergillus flavus* and *Aspergillus niger.*

| Fermentation Periods (day) | TTA Content(mg/mL) |
|---|---|
| 1 | $15.76\pm1.88^{b}$ |
| 2 | $6.66\pm1.48^{a}$ |
| 3 | $10.50\pm0.86^{ab}$ |
| 4 | $8.06\pm1.42^{ab}$ |
| 5 | $9.00\pm4.76^{ab}$ |
| 6 | $13.16\pm2.12^{ab}$ |
| 7 | $13.00\pm2.07^{ab}$ |

Values are means of triplicate readings and SEM of TTA of fermented plantain peel, Values along the column having different superscripts are significantly different at ($p<0.05$).

## Reducing Sugar of Fermented Plantain Peel by *Aspergillus flavus* and *Aspergillus niger.*

The highest reducing sugar (3.26 ± 0.01 mg/mL) was obtained on day 2 by *Aspergillus flavus* and *Aspergillus niger* while the lowest value of reducing sugar (0.38 ± 0.01 mg/mL) was obtained on day 7 as shown in Table 7.

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**Table 7:** Reducing Sugar Content of Fermented Plantain Peel by *Aspergillus flavus* and *Aspergillus niger* at Different Fermentation Periods.

| Fermentation Period (day) | Reducing Sugar Content (mg/mL) |
|---|---|
| 1 | $1.92 \pm 0.02^{e}$ |
| 2 | $3.26 \pm 0.01^{f}$ |
| 3 | $1.92 \pm 0.01^{e}$ |
| 4 | $1.35 \pm 0.03^{c}$ |
| 5 | $0.92 \pm 0.02^{b}$ |
| 6 | $1.64 \pm 0.02^{d}$ |
| 7 | $0.38 \pm 0.01^{a}$ |

**Effect of Fermentation Period for Itaconic Acid Production by *Aspergillus flavus* and *Aspergillus niger***

The highest concentration of Itaconic acid (9.43±0.02 mg/mL) was produced on day 2 by both *Aspergillus flavus* and *Aspergillus niger* while the lowest concentration (1.25±0.01 mg/mL) was obtained on day 5 as shown in Table 8

**Table 8:** Itaconic Acid Contents of Fermented Plantain Peel by *Aspergillus flavus* and *Aspergillus niger.*

| Fermentation Period (day) | Itaconic Acid Content (mg/mL) |
|---|---|
| 1 | $2.06 \pm 0.00^{c}$ |
| 2 | $9.43 \pm 0.02^{g}$ |
| 3 | $7.35 \pm 0.00^{f}$ |
| 4 | $3.03 \pm 0.01^{e}$ |
| 5 | $1.25 \pm 0.01^{a}$ |
| 6 | $2.39 \pm 0.01^{d}$ |
| 7 | $1.34 \pm 0.20^{b}$ |

Values are means of duplicate readings and SEM of itaconic acid concentration of fermented plantain peel, Values within the column having different superscripts are significantly different ($p<0.05$).

## DISCUSSION

Results obtained from the analysis carried out in this study showed that the carbohydrate content was the highest of all the extract indicating that the plantain peel is a good source of fermentable sugars that can be metabolized by fungi during the fermentation process to produce metabolites such as itaconic acid. These results accord with other works that revealed the ability of plant-based substrates for the fungal fermentation (Agboola *et al*., 2016; Oboh *et al*., 2017). The moisture analysis indicates that the plantain peel can indeed be used for solid state fermentation without experiencing severe loss of moisture or contamination. Also, the moderate ash content indicated that the plantain peel contains some minerals required by microorganism for growth (Adeleke *et al*., 2020).

The physiochemical composition of the plantain peel before fermentation (Table 2) indicates a moderate acidic environment, which is favorable for fungal fermentation. Similar pH values have been reported in studies where plantain peel was used as a fermentation substrate (Adeleke *et al.,* 2020). The titratable acidity reflects the organic acid content, which could act as a buffer during fermentation, thereby supporting microbial activity.

Following the fermentation process, fungal isolates were characterized and the most frequent strains reported as *Aspergillus niger* and *Aspergillus flavus*. *Aspergillus. niger* better produces organic acid like itaconic acid because of its ability to reduce complex substrate such as lignocellulose and cellulose (Benkerroum, 2016). Likewise, *A. flavus* has been identified to synthesize set of bioactive compounds, which put this fungal species in a good position to offer high add-on value metabolites. This also aligns with findings by Nyongesa *et al*. (2015), who identified *A. niger* as an excellent producer of enzymes that degrade plant materials. *Aspergillus flavus* is also known for its abilities to produce organic acids, making it a suitable candidate for itaconic acid production.

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The pH influences nutrient solubility and activity of enzymes engaged in substrate catabolism, which influences the general efficiency of fermentation. The pH values (Table 5) fluctuated during fermentation, with the lowest pH on day 1 and the highest on day 5. This change in pH reflects the balance between acid production and consumption during fermentation. Research by Chen *et al.* (2020) supports this observation, noting that pH levels tend to decrease initially due to organic acid production, followed by a slight increase as fermentation stabilizes. In agreement with the results of the current work, Hussain *et al*. (2017) pointed out that acidic conditions support the synthesis of organic acids by fungi.

Total titratable acidity (Table 6) peaked on day 1 and decreased thereafter. This high initial acidity could be as a result of the rapid production of organic acids in the early stages of fermentation. An identical trend was noticed by Gao *et al*. (2019), where the titratable acidity was highest at the beginning of fermentation due to the active metabolism of the fungi.

The highest reducing sugar content using *Aspergillus flavus* and *Aspergillus niger* was observed by on day 1. The lowest reducing sugar content was obtained on day 2 (Table 7). These results indicate that extended fermentation periods enhance the breakdown of carbohydrates into reducing sugars, which is a precursor step for itaconic acid production (Kumar *et al*., 2017). These findings suggest that TTA levels correlate with fermentation duration, impacting the efficiency of acid production (Sriariyanun *et al*., 2019). This outcome supports other work showing that *Aspergillus* spp possesses enhanced capabilities of fermentation of substrates to organic acids than other species of fungi (Hussain *et al*., 2017).

The findings stated showed that duration had a positive correlation with the yield of itaconic acid, up to a certain point. Inoculum age is related to higher fermentation rates since the fungus population grows rapidly and there is enhanced production of enzymes when breaking down the substrate (Benkerroum, 2016). This is in tandem with the earlier emerging research that showed that the enhancement of fermentation time created the necessary conditions for substrates' complete utilization and formation of metabolites (Hussain *et al*., 2017).

With respect to the yield of itaconic acid, the studies showed that the yield was substrate dependent. With the high carbohydrate content of plantain peel, reasonable yield of itaconic acid was obtained. This result supports literature that indicates plant biomass as a viable feedstock to produce organic acids through fungal fermentation (Agboola *et al*., 2016). In terms of itaconic acid concentration (Table 4), the highest concentration was recorded on day 2, which then declined by day 5. This pattern could be due to the exhaustion of fermentable sugars and the accumulation of secondary metabolites that inhibit further production. Studies by Ajiboye *et al*. (2018) corroborate this finding, indicating that peak itaconic acid production typically occurs within the first few days of fermentation, after which the production rate decreases. The results of this study demonstrate that plantain peel is a suitable substrate for itaconic acid production, with *Aspergillus niger* and *Aspergillus flavus* effectively fermenting the peel to produce itaconic acid. The findings are consistent with recent research, indicating that optimizing fermentation conditions can enhance yield and efficiency in future studies. This work enlightens the possibility of using plantain peel at low cost, to enhance the yield of itaconic acid through solid-state fermentation (Hussain *et al*., 2017).

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## Conclusion

This study has demonstrated that plantain peel is a promising substrate to produce itaconic acid using *Aspergillus flavus* and *Aspergillus niger* obtained as resident fungi from fermentation of plantain peel. The proximate composition analysis showed that plantain peel has the right composition to serve as a good substrate in industries, especially in solid state fermentation (S.S.F) processes. These consistencies in composition and low moisture content increase stability for fermentation and thus a good substrate for synthesizing high value biochemicals such as itaconic acid.

## Recommendation

From the findings of this study, it is recommended that future research could focus on optimizing fermentation conditions, such as temperature, inoculum size and substrate concentration, to enhance itaconic acid yield. Utilizing mutant strains of *Aspergillus niger* and *Aspergillus flavus* may also improve production efficiency. Additionally, exploring co-culturing with other microorganisms capable of producing organic acids could enhance sugar utilization and acid production

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