CITRIC ACID PRODUCTION FERMENTATION PROCESS

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ABSTRACT

Citric acid is the most important organic acid produced in tonnage and is extensively used in food and pharmaceutical industries. It is produced mainly by submerged fermentation using Aspergillus niger *or* Candida *sp. from different sources of carbohydrates, such as molasses and starch based media. However, other fermentation techniques, e.g. solid state fermentation and surface fermentation, and alternative sources of carbon such as agroindustrial residues have been intensively studied showing great perspective to its production. This paper reviews recent developments on citric acid production by presenting a brief summary of the subject, describing microorganisms, production techniques, and substrates, etc.*

Keywords: *Citric acid production, Aspergillus niger, surface fermentation, solid state fermentation,*

Submerged fermentation

1. INTRODUCTION:

Citric acid fermentation was first observed as a fungal product by Wehmer in 1893 by a culture of *Penicillium glaucum* on sugar medium. After a few years, he isolated two new fungal strains with the ability to accumulate citric acid, which were designated *Citromyces (Penicillium)*. However, industrial trials did not succeed due to contamination problems and long duration of fermentation. It was the work of Currie which opened up the way for successful industrial production of citric acid. In 1916, he found that numerous strains of *Aspergillus niger* produced significant amounts of citric acid. The most important finding was that *Aspergillus niger* grew well at pH values around 2.5–3.5 and high concentrations of sugars favour citric acid production. [1]

The first citric acid fermentations were carried out in surface cultures. In the 1930s, some units were implanted in England, in Soviet Union, and in Germany for the commercial production. In general, citric acid is commercially produced by submerged microbial fermentation of molasses; the fermentation process using *Aspergillus niger* is still the main source of citric acid worldwide. Although methods were well developed to synthesise citric acid using chemical means, better successes were achieved using microbial fermentations, and over the period of time, this technique has become the method of ultimate choice for its commercial production over chemical synthesis. Despite that, the introduction of submerged fermentation presented several problems, including the choice of productive strains with low sensitivity to trace elements. It was necessary to consider raw material much more carefully. Several works were dedicated to the optimization of the conditions for the utilization of cheap material like sugar cane molasses, beet molasses, starch and hydrolysate starch. Various processes for treating and purifying molasses were developed, especially for the removal of trace metals. Moreover, it was found that a small excess of copper ions was beneficial to achieve high yields of citric acid. [2]

There are annual growths of 3.5–4.0 % in demand/ consumption of citric acid. In the last years, a considerable interest has been shown in using agricultural products as alternative sources of carbon and their wastes such as maize, apple and grape pomace, pineapple, mandarin orange and brewery wastes, citrus and kiwi fruit peel for citric acid production by Aspergillus niger. The industry is seeking newer cheap and economic process technology.

2. APPLICATIONS OF CITRIC ACID:

Citric acid is mainly used in food industry because of its pleasant acid taste an its high solubility in water. It is worldwide accepted as “GRAS” (generally recognized as safe), approved by the Joint FAO/WHO Expert Committee on Food Additives. The pharmaceutical and cosmetic industries retain 10% of its utilization and the remainder is used for various other purposes. Table 1 presents main applications of citric acid.

Table 1: Applications of Citric Acid

Industry	**Applications**
Beverages	Provides tartness and complements fruits and berries flavours. Increases the effectiveness of antimicrobial preservatives. Used in pH adjustment to provide uniform acidity.
Jellies, Jams and Preserves	Provides tartness. pH adjustment.
Candy	Provides tartness. Minimizes sucrose inversion. Produces dark colour in hard candies. Acts as acidulant.
Frozen fruit	Lowers pH to inactivate oxidative enzymes. Protects ascorbic acid by inactivating trace metals
Dairy products	As emulsifier in ice creams and processed cheese; acidifying agent in many cheese products and as an antioxidant.
Fats and oils	Synergist for other antioxidants, as sequestrate.
Pharmaceuticals	As effervescent in powders and tablets in combination with bicarbonates. Provides rapid dissolution of active ingredients. Acidulant in mild astringent formulation. Anticoagulant.
Cosmetics and toiletries	pH adjustment, antioxidant as a metallic ion chelator, buffering agent.
Industrial applications	Sequestrant of metal ions, neutralizing, buffer agent
Metal cleaning	Removes metal oxides from surface of ferrous and nonferrous metals, for preparational and operational cleaning of iron and copper oxides

3. MICRO-ORGANISMS USED FOR CITRIC ACIC PRODUCTION:

A large number of micro-organisms including bacteria, fungi and yeasts have been employed to produce citric acid. Most of them, however, are not able to produce commercially acceptable

yields. This fact could be explained by the fact that citric acid is a metabolite of energy metabolism and its accumulation rises in appreciable amounts only under conditions of drastic imbalances. The strains reported to produce citric acid. Table 2 shows the micro-organisms used to produce citric acid. Among these, only *A. niger* and certain yeasts such as *Saccharomycopsis* sp. are employed for commercial production. However, the fungus *A. niger* has remained the organism of choice for commercial production. The main advantages of using this microorganism are: (a) its ease of handling, (b) its ability to ferment a variety of cheap raw materials, and (c) high yields. [3]

Table 2: Micro-organisms employed for citric acid production

Micro-organisms	**References**
Fungi	
Aspergillus niger	Hang & Woodams 1984, 1985, 1987, Roukas 1991, Garg & Hang 1995, Pintado 1998, Vandenberghe 1999
A. aculeatus	El Dein & Emaish, 1979
A. awamori	Grewal & Kalra, 1995
A. carbonarius	El Dein & Emaish, 1979
A. wentii Karow & Waksman, 1947	Karow & Waksman, 1947
A. foetidus	Chen, 1994; Tran et al., 1998
Penicillium janthinelum	Grewal & Kalra, 1995
Yeasts	
Candida tropicalis	Kapelli et al., 1978
C. oleophila	Ishi et al., 1972
C. guilliermondii	Miall & Parker, 1975; Gutierrez et al., 1993
C. parapsilosis	Omar & Rehm, 1980
C. citroformans	Uchio et al., 1975
Hansenula anamola	Oh et al., 1973
Bacteria	
Bacillus	*licheniformis* Sardinas, 1972

Arthrobacter paraffinens	Kroya Fermentation Industry, 1970
Corynebacterium sp.	Fukuda et al., 1970 T

4. PRODUCTION TECHNIQUES AND RAW MATERIALS:

Although citric acid is mostly produced from starch or sucrose based media using liquid fermentation, a variety of raw materials such as molasses, several starchy materials and hydrocarbons have also been employed. Classified raw materials used for citric acid production in to two groups: [4]

I. Raw materials with a low ash content from which the cations could be removed by standard procedures (e.g. cane or beet sugar, dextrose syrups and crystallized dextrose);
II. Raw materials with a high ash content and high amounts of other non-sugar substances (e.g. cane and beet molasses, crude unfiltered starch hydro-lysates).

Several attempts have been made to produce citric acid using molasses, which is preferred due its low cost and high sugar content (40-55%). The composition of molasses depends on various factors, e.g. the kind of beet and cane, methods of cultivation of crops and fertilizers and pesticides applied during cultivation, conditions of storage and handling (e.g. transport, temperature variations), production procedures, etc. Both, cane and beet molasses are suitable for citric acid production. However, beet molasses is preferred due to its lower content of trace metals. Generally, cane molasses contains calcium, magnesium, manganese, iron and zinc, which have a retarding effect on the synthesis of citric acid. Consequently, some pre-treatment is required for the removal/reduction of trace metals. Despite that, cane molasses possesses difficulties in achieving good fermentation yields.

Various other agro-industrial residues such as apple pomace, cassava bagasse, coffee husk, wheat straw, pineapple waste, sugar beet cosset, kiwi fruit peel, etc. have been investigated with solid state fermentation techniques for their potential to be used as substrates for citric acid production. In fact, these residues are very well adapted to solid-state cultures due to their cellulosic and starchy nature. However, despite the fact that these solid residues provide rich nutrients to the micro-organisms, and are good substrates for growth and activity of micro-organisms, much remains to be done for developing commercially feasible process utilizing these residues. [5]

4.1. Submerged Fermentation:

The submerged fermentation (SmF) process is the commonly employed technique for citric acid production. It is estimated that about 80% of world production is obtained by SmF. Several advantages such as higher yields and productivity and lower labour costs are the main reasons for this. Two types of fermenters, conventional stirred fermenters and tower fermenters are employed, although the latter is preferred due to the advantages it offers on price, size and operation. Preferentially, fermenters are made of high grade steel and require provision of aeration system, which can maintain a high dissolved oxygen level. Fermenters for citric acid production do not have to be built as pressure vessels since sterilization is performed by simply steaming without applying pressure. Cooling can be done by an external water film over the entire outside wall of the fermenter.

Molasses and other raw materials demand pre-treatment, addition of nutrients and sterilization. Inoculation is performed either by adding a suspension of spores, or of pre-cultivated mycelia. When spores are used, a surfactant is added in order to disperse them in the medium. For pre-cultivated mycelia, an inoculum size of 10% of fresh medium is generally required. Normally, submerged fermentation is concluded in 5 to 10 days depending on the process conditions. It can be carried out in batch, continuous or fed batch systems, although the batch mode more frequently used. [5]

Table 3: Raw materials and micro-organisms employed in submerged fermentation for citric acid production

Raw material	Strain	Citric acid	Yield, %	References
Brewery wastes	*A. niger* ATTC 9142	19 g/L	78.5	Roukas & Kotzekidou, 1986
Beet molasses	*A.niger* ATTC 9142 *Yarrow lipolytica* A101	109 g/L 54 g/L	- 68.7	Ogawa & Fazeli, 1976 Kautola et al., 1992
Cane molasses	*A. niger* T 55	-	65	Kundu et al, 1984
Wood Hemicellulose	*A. niger* IMI- 41874 *S. lipolytica* IFO 1658	27 g/L 9 g/L	45 41	Maddox et al., 1985 Maddox et al., 1985
Date syrup	*A. niger* ATTC 9142	-	50	Roukas & Kotzekidou,

				1997
Corn starch	*A. niger* IM-155	-	62	Nguyen et al., 1992
Starch hydrolysate	*Y. lipolytica* DS-1 *Y. lipolytica* A-101	- -	- 75	Shah et al., 1993 Wojtatowicz et al., 1993
Rapeseed oil	*Y. lipolytica* A-101	-	57	Wojtatowicz et al., 1993
Soybean oil	*Y. lipolytica* A-101	-	63	Wojtatowicz et al., 1993
Coconut oil	*C.lipolytica* N-5704	-	99.6	Ikeno et al., 1975
Palm oil	*C.lipolytica* N-5704	-	155b	Ikeno et al., 1975
Olive oil	*C.lipolytica* N-5704	-	119	Ikeno et al., 1975
Soybean oil	*C.lipolytica* N-5704	-	115	Ikeno et al., 1975
Glycerol	*C.lipolytica* N-5704	-	58.8	Ikeno et al., 1975
n-Paraffin	*C.lipolytica* N-5704	-	161	Ikeno et al., 1975

4.2. Surface Fermentation:

The first individual process for citric acid production was the liquid surface culture (LSC), which was introduced in 1919 US. After that, other methods of fermentation, such as submerged fermentation were developed. Although this technique is more sophisticated, surface method required less effort in operation and installation and energy cost. In the classical process for citric acid manufacture, the culture solution is held in shallow trays (capacity of 50-100 L) and the fungus develops as a mycelial mat on the surface of the medium. The trays are made of high purity aluminium or special grade steel and are mounted one over another in stable racks. The fermentation chambers are provided with an effective air circulation in order to control temperature and humidity. Fermentation chambers are always in aseptic conditions, which might be conserved principally during the first two days when spores germinate. Frequent contamination is mainly caused by Penicillin, other Aspergilli, yeast and lactic bacteria. Refined or crude sucrose, cane syrup or beet molasses are generally used as sources of carbon. When applied, molasses is diluted to 15-20% and is treated with hex cyanoferrate (HFC). [6]

4.3. Solid-state Fermentation:

Solid-state fermentation (SSF) has been termed as an alternative method to produce citric acid from agro-industrial residues. Citric acid production by SSF (Koji process) was first developed in Japan and is as the simplest method for its production. SSF can be carried out using several raw materials. Generally, the substrate is moistened to about 70% moisture depending on the substrate absorption capacity. The initial pH is normally adjusted to 4.5-6.0 and the temperature of incubation can vary from 28 to 30°C. The most commonly organism is *Aspergillus niger*. However there also have been reports with yeasts. One of the important advantages of SSF process is that the presence of trace elements may not affect citric acid production as harmfully as it does in SmF. Consequently, substrate pre-treatment is not required. [7]

Different types of fermenters such as conical flasks, glass incubators and trays, etc. have been used for citric acid fermentation in SSF. Used Erlenmeyer flasks and glass columns for the production of citric acid from gelatinized cassava bagasse. Higher yields were obtained in flasks without any aeration, and very little sporulation was observed. The same yields were found in column reactors only with variable aeration. This showed great perspective to use SSF process for citric acid production in simple tray type fermenters. [8]

Table 4: Raw materials and micro-organisms employed in solid state fermentation for citric acid production

Raw material	Strain	Citric acid	Yield(%)	Reference
Apple pomace	*A.niger NRRL2001* *NRRL 2270* *NRRL 599* *NRRL 328* *NRRL 567*	766 g/kg 816 g/kg 771 g/kg 798 g/kg 883 g/kg		Hang & Woodams, 1984
Grape pomace	*A.niger NRRL2001* *NRRL 2270* *NRRL 599* *NRRL 328* *NRRL 567*	413 g/kg 511 g/kg 498 g/kg 523 g/kg 600 g/kg	88	Hang & Woodams, 1985
Kiwifruit peel	*A .niger NRRL 567*	100 g/kg		Hang & Woodams, 1987
Cellulose hydrolysate and Sugar cane	*A. niger*	29 g/kg	44	Mannomani & Sreekantiah, 1987

Orange waste	*A. niger*	46 g/kg		Aravantinos-Zafiris et al., 1994
Beet molasses (Ca-alginate gel)	*A.niger ATCC 9142*	35 g/kg		Roukas, 1991
Saccharose (Sugar cane bagasse)	*A. niger CFTRI 30*	174 g/kg		Shankaranand & Lonsane, 1993
Coffee husk	*A. niger CFTRI 30*	150 g/kg		Shankaranand & Lonsane, 1994
Okara (soy residue)	*A. niger*	96 g/kg		Khare et al., 1995
Pineapple waste	*A.niger ATCC 1015* *A.niger ACM 4942*	132 g/kg 194 g/kg	74	Lima et al., 1995 Tran et al., 1998
Glucose (Sugar cane bagasse)	*A.niger CBS733.88*	21.24 g/kg		Pallares et al., 1996
Mussel processing wastes (polyurethane foams)	*A. niger*	300 g/kg		Pintado et al., 1998
Cassava bagasse	*A. niger LPB-21*	347 g/kg	67	Vandenberghe et al., 1999

5. FACTORS AFFECTING CITRIC ACID PRODUCTION:

5.1. Medium and its components:

Carbon source:

Citric acid accumulation is strongly affected by the nature of the carbon source. The presence of easily metabolized carbohydrates has been found essential for good production of citric acid. That sucrose was the most favourable carbon source followed by glucose, fructose and galactose. Galactose contributed to a very low growth of fungi and did not favour citric acid accumulation. Other sources of carbon such as sorbose, ethanol, cellulose, manitol, lactic, malic and acetoglutaric acid, allow a limited growth and low production. Starch, pentoses (xyloses and arabinoses), sorbitol and pyruvic acid slow down growth, though the production is minimal.

According initial sugar concentration was critical for citric acid production and other organic acids produced by *Aspergillus niger*. That *Aspergillus niger* strains needed an initial sugar concentration of 10-14% as optimal; no citric acid was produced at sugar concentration of less than 2.5%. That immobilized cells of *Aspergillus niger* needed lower concentrations of sucrose than free cells culture, in order to obtain high yields (200 g of citric acid/L for free cells culture, and 120 g/L for immobilized cells). The influence of different sources of carbon on citric acid production by *Aspergillus niger* and *Saccharomycopsis lipolytica*. Glucose, maltose, galactose, xylose and arabinose were tested. Fermentation was carried out in 8 and 4 days, respectively, at 30°C and 180 rpm. Better results were found for *Aspergillus niger* with 0.45 g of citric acid/ g of glucose corresponding to 27 g/L. *Saccharomycopsis lipolytica* produced 0.41 g/g of glucose or 9 g/L which was not so bad.

As presented previously, several raw materials can be employed successfully for citric acid production. There are some critical factors (costs, need of pre-treatment), which should be considered for substrate determination. One another aspect is the presence of trace elements, which can act as inhibitors or stimulants. Consequently, sometimes it is necessary to conduce a pre-treatment, e.g.; precipitation of trace metals of molasses by potassium ferrocyanide. [8]

Nitrogen source:

Citric acid production is directly influenced by the nitrogen source. Physiologically, ammonium salts are preferred, e.g. urea, ammonium sulfate, ammonium chlorure, peptone, malt extract, etc. Nitrogen consumption leads to pH decrease, which is very important point in citric acid fermentation. However, it is necessary to maintain pH values in the first day of fermentation prior to a certain quantity biomass production. Urea has a tampon effect, which assures pH control. The concentration of nitrogen source required for citric acid fermentation is 0.1 to 0.4 N/liter. A high nitrogen concentration increases fungal growth and the consumption of sugars, but decreases the amount of citric acid produced. [9]

Phosphorous source:

Presence of phosphate in the medium has a great effect on the yield of citric acid. Potassium dihydrogen phosphate has been reported to be the most suitable phosphorous source. The phosphorous at concentration of 0.5 to 5.0 g/L was required by the fungus in a chemically defined medium for maximum production of citric acid. Phosphate is known to be essential for the growth and metabolism of *Aspergillus niger*. Low levels of phosphate favour citric acid production, however, the presence of excess of phosphate was shown to lead to the formation of certain sugar acids, a decrease in the fixation of CO_2, and the stimulation of growth. Phosphates acts at the level of enzyme activity and not at the level of gene expression. It is interesting to note that different strains require distinct nitrogen and phosphorous concentrations in the medium. In fact, nitrogen and phosphorous limitation is a crucial factor in citric acid production as there is an interaction between them. Consequently, the study of their combined effect is necessary. The culturing modality conditions the behaviour of the micro-organisms referring to the tendencies of production as a function of the levels of N and P. The author used as first order an empirical model based on rotatable design to study the effect of both nutrients. As expected, for the two studied strains, a similar behaviour was noticed, showing an improvement towards low levels of N and P in submerged culture, and toward high levels in solid state culture, and with superior productions for the last one. The specificity of solid state culture is largely due to a lower diffusion rate of nutrients and metabolites, which occurs in low water activity conditions. Consequently, a strain with large requirements of N and P seems to be disfavoured, due to the restriction of accessibility to the nutrients in the medium. [10]

Trace elements:

Trace element nutrition is probably the main factor influencing the yield of citric acid. A number of divalent metals such as zinc, manganese, iron, copper and magnesium have been found to affect citric acid production by *Aspergillus niger*. However, it is crucial to take into account the interdependence of medium constituents in SmF and, probably, in SSF. Zinc favoured the production of citric acid if added with KH_2PO_4. On the other hand, the presence of manganese ions and iron and zinc (in high concentrations) could cause the reduction of citric acid yields only in phosphate free medium. There were few differences in the response of *Aspergillus niger* to metal ions and minerals in SSF and in SmF systems. SSF systems were able to overcome the adverse effects of the high concentrations of these components in the medium. As a consequence of this, the addition of chelating agents such as potassium ferrocyanide to the medium proved to be of no use.

Copper was found to complement the ability of iron at optimum level, to enhance the biosynthesis of citric acid. Manganese deficiency resulted in the repression of the anaerobic and TCA cycle enzymes with the exception of citrate synthetase. This led to overflow of citric acid as product of glycolysis. A low level of manganese (ppm) was capable to reduce the yield of citric acid by 10%. Citric acid accumulation decreased by the addition of iron, which also had some effect on mycelial growth. The presence of different copper concentrations in the pellet formation medium was very important in order to enhance a suitable structure, related to cellular physiology, for citric acid production. The optimal initial $CuSO_4.5H_2O$ concentration was 78 mg/L. Magnesium is required both for growth as well as for citric acid production. Optimal concentration of magnesium sulfate was found in the range of 0.02-0.025%. [11]

Lower alcohols:

Addition of lower alcohols enhances citric acid production from commercial glucose and other crude carbohydrate. Appropriate alcohols are methanol, ethanol, isopropanol or methyl acetate. The optimal amount of methanol/ethanol depends upon the strain and the composition of the medium, generally optimum range being 1-3%.

The addition of ethanol resulted in two-fold increase in citrate synthetase activity and 75% decrease in aconitase activity. Whereas the activities of other TCA cycle enzymes increased slightly. They also found that coconut oil influenced citric acid production in a sucrose medium when added at 3% (v/w). Alcohols have been shown to principally act on membrane permeability in micro-organisms by affecting phospholipid composition on the cytoplasmatic membrane. The alcohols stimulate citric acid production by affecting growth and sporulation through the action not only on the cell permeability but also the spatial organization of the membrane, or changes in lipid composition of the cell wall. [12]

5.2. Process parameters:

pH:

The pH of a culture may change in response to microbial metabolic activities. The most obvious reason is the secretion of organic acids such as citric, acetic or lactic acids, which will cause the pH to decrease. Changes in pH kinetics depend highly also on the micro-organism. With *Aspergillus* sp., *Penicillium* sp. and *Rhizopus* sp., pH can drop very quickly until less than 3.0. For other groups of fungi such as *Trichoderma*, *Sporotrichum*, *Pleurotus* sp., pH is more stable (between 4 and 5). Generally, a pH below 2.0 is required for optimum production of citric acid. A low initial pH has the advantage of checking contamination and inhibiting oxalic acid formation. A pH of 2.2 was reported to be optimum for the growth of the mould as well as for the production of citric acid whereas; a higher pH i.e. 5.4 and 6.0-6.5 has been found optimum for citric acid production in molasses medium. [13]

Aeration:

Aeration has been shown to have a determinant effect on citric acid fermentation. Increased aeration rates led to enhanced yields and reduced fermentation time. The influence of dissolved oxygen concentration on citric acid formation has been examined. It is important to maintain the oxygen concentration above 25% saturation and interruptions in oxygen supply may be quite harmful. The high demand of oxygen is fulfilled by constructing appropriate aeration devices, which is also dependent on the viscosity of the fermentation broth. This is an additional reason why small compact pellets are the preferred mycelial forms of *Aspergillus niger* during fermentation. When the organism turns into filamentous developments, e.g. due to metal contamination, the dissolved oxygen tension rapidly falls to less than 50% of its previous value, even if the dry weight has not increased by more than 5%. Aeration is performed during the whole fermentation with the same intensity through the medium at a rate of 0.5 to 1.5 vvm. However, because of economic reasons, it's usually preferred to start with a low aeration rate (0.1 to 0.4 vvm). High aeration rates lead to high amounts of foam, especially during the growth phase. Therefore, the addition of antifoaming agents and the construction of mechanical "deformers" are required to tackle this problem. [14]

6. PRODUCT RECOVERY:

The recovery of citric acid from liquid fermentation is generally accomplished by three basic procedures, precipitation, extraction, and adsorption (mainly using ion exchange resins). Citric acid extraction has been described by the Food and Drug Administration (1975) of the United States. [15] Citric acid extracted by this method has been recommended suitable for use in food and drugs. Precipitation is the classical method and it is performed by the addition of calcium oxide hydrate (milk of lime) to form the slightly soluble tri-calcium citrate tetra hydrate. The precipitated tri-calcium citrate is removed by filtration and washed several times with water. It is then treated with sulphuric acid forming calcium sulphate, which is filtered off. Mother liquor containing citric acid is treated with active carbon and passed through cation and anion exchangers. Several anion-exchange resins are commercially available. Finally, the liquor is concentrated in vacuum crystallizers at 20-25°C, forming citric acid monohydrate. Crystallization at temperatures higher to this is used to prepare anhydrous citric acid. [16]

7. CONCLUSIONS:

Since the beginning of this century, citric acid production has been intensively studied and great alternatives to this process have been found to follow its great demand. The use of alternative raw materials to produce citric acid by SmF, LSC, and SSF seems to be a suitable possibility. However, it is necessary to adapt the right type of raw material to the right technique e.g. cassava bagasse employed as substrate in SSF, or cellulose hydrolysate used in SmF. The need of some pre-treatment of raw materials may enhance the fermentation efficiency. One area, which needs attention is the development of continuous culture techniques which have been attempted but only at the laboratory scale. Another area is the strain improvement with improved substrate utilization efficiency.

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