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Improved astaxanthin production by *Xanthophyllomyces dendrorhous* growing on enzymatic wood hydrolysates containing glucose and cellobiose

J. M. Cruz & J. C. Parajó*

Department of Chemical Engineering, University of Vigo (Campus Orense), Faculty of Science, Las Lagunas, 32004 Orense, Spain

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Eucalyptus wood samples were treated with NaOH solutions and subjected to enzymatic hydrolysis with a commercial, β-glucosidase-deficient, cellulase complex or with a mixture of cellulase and β-glucosidase. Hydrolysates containing glucose or glucose and cellobiose were supplemented with KNO_3 and nutrients, and used as culture media for the proliferation of *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*) ATCC 24228. For comparative purposes, similar experiments were carried out with media made from commercial sugars. All the bioconversion experiments were carried out in a batch fermenter. At selected fermentation times, the concentrations of substrate (or substrates), biomass and carotenoids were determined. Under the best conditions assayed (hydrolysates obtained with cellulases supplemented with an inorganic nitrogen source), biomass concentrations in the vicinity of 10 g litre^{-1}, with volumetric carotenoid concentration of 2.14 mg litre^{-1}, were reached after 74 h of fermentation. These results compare favourably with those reported for carotenoid production from enzymatic hydrolysates containing glucose as the sole carbon source.

INTRODUCTION

The biotechnological production of carotenoids such as astaxanthin is a research field with increasing interest, owing to their high market price and growing demand. The utilization of astaxanthin as a component of rations for salmonids or poultry for improving the colouration of flesh or egg yolks is well known (Johnson *et al.*, 1980*a*,*b*; Johnson and An, 1991; Dike *et al.*, 1992; Tangeras and Slinde, 1994).

The biotechnological production of carotenoids can be based on the utilization of algae, bacteria or yeast. The red yeast *Phaffia rhodozyma* (or *Xanthophyllomyces dendrorhous*) is one of the most promising micro-organisms for the commercial production of astaxanthin. The propensity of *Phaffia* yeasts for growing on a variety of carbon sources, such as glucose, cellobiose, maltose, sucrose, lactose, xylose and arabinose (Johnson and An, 1991; Fang and Cheng, 1993; Parajó *et al.*, 1997*a*,*b*), is a remarkable advantage. Three of the above sugars can be obtained from *Eucalyptus* wood: the hydrolysis of its hemicellulosic fraction (rich in xylan, a polymer built from xylose units) leads to xylose solutions, whereas the enzymatic hydrolysis of its cellulosic fraction leads to glucose or glucose–cellobiose mixtures (depending on the relative β-glucosidase activity of the enzymatic complex).

Obvious economic advantage can be achieved by formulating culture media from processed plants or lignocellulosic materials instead of from commercial sugars. In this field, proliferation of *Phaffia* has been assayed in media made from plant or fruit juices (Okagbue and Lewis, 1984; Meyer and du Preez, 1994; Fontana *et al.*, 1996), byproducts of wet corn milling (Hayman *et al.*, 1995), molasses (Haard, 1988), acid hydrolysates of lignocellulosic materials (Martin *et al.*, 1992, 1993; Acheampong and Martin, 1995) and wood enzymatic hydrolysates (Parajó *et al.*, 1997*c*). In a previous study (Parajó *et al.*, 1997*c*), the authors considered the proliferation of *Phaffia* in shaken flasks using media made from enzymatic hydrolysates of a processed softwood. The enzymatic complex utilized in these assays was a mixture of cellulases (from *Trichoderma reesei*)

*To whom correspondence should be addressed. Fax: 00-34-88-387001; e-mail: jcparajo@uvigo.es

and β-glucosidase (from *Aspergillus niger*). Under these conditions, glucose was the sole sugar produced in hydrolysis, and the media made from hydrolysates were easily utilized by *Phaffia*.

Johnson and Lewis (1979) reported that *P. rhodozyma* UCD 67-210 cells grown on cellobiose solutions showed high pigmentation. Martin *et al.* (1993) observed an improved carotenogenesis in *Phaffia* cells cultured on media made from peat hydrolysates, and suggested that the presence of cellobiose in the fermentation media could be responsible for this finding. Cellobiose-containing culture media can be easily made from lignocellulose enzymatic hydrolysates: cellobiose is an intermediate of the cellulose depolymerization by cellulases (Phillips and Humphrey, 1982), and cellobiose concentrations of several g litre^{-1} can be achieved by using enzymatic complexes with low β-glucosidase activity in the hydrolysis step. With this operational procedure, glucose is also present in the hydrolysis media.

Based on these ideas, *Eucalyptus* wood (a cheap and largely available hardwood with favourable characteristics for chemical processing) was selected as a raw material in this work. A single alkaline treatment under selected conditions led to a solid residue susceptible to enzymic saccharification. In order to obtain solutions containing both glucose and cellobiose, solid residues from treatments were subjected to enzymatic hydrolysis with a commercial cellulase complex deficient in β-glucosidase activity. For comparative purposes, additional β-glucosidase (from *A. niger*) was added to the hydrolysis media in selected experiments. After supplementation with nutrients and with an inorganic nitrogen source, hydrolysates were used as culture media for the proliferation of *X. dendrorhous* (*P. rhodozyma*) ATCC 24228. Additional assays were made using culture media formulated with commercial sugars. The dynamics of the concentrations of substrates, biomass and carotenoids were determined, and the experimental results compared with reported data.

MATERIALS AND METHODS

Raw material and chemical processing

Eucalyptus globulus wood chips were collected from local industries, milled to pass a 1 mm screen, homogenized in a single lot and stored. Samples from the above lot were subjected to quantitative saccharification with sulphuric acid using standard methods (Browning, 1967) in order to establish their content of cellulose, hemicelluloses and lignin. Lignin was determined gravimetrically as the acid-insoluble residue, whereas cellulose and hemicelluloses were estimated by high performance liquid chromatography (HPLC) determination of the sugars present in liquors (Parajó *et al.*, 1995). In order to obtain substrates susceptible to enzymatic hydrolysis, wood samples were submitted to alkaline treatments under selected operational conditions (see below). Processed wood samples were analysed for cellulose, hemicellulose and lignin using the same methods employed for untreated wood.

Enzymatic hydrolysis

The solid residues from alkaline treatments were hydrolysed in media containing commercial cellulases from *T. reesei* ('Celluclast' enzymes kindly provided by Novo, Denmark). In selected experiments, β-glucosidase from *A. niger* ('Novozym', Novo) was added to the hydrolysis media. The cellulase activity was fixed in 1.5 Filter Paper Units ml^{-1}. In experiments with media enriched in β-glucosidase, this enzymatic activity was kept at 4 IU ml^{-1}. The enzymatic assays were performed during 48 h at 48.5°C and pH 4.85 (citrate buffer 0.05 N) using a liquor/solid ratio of 10 g g^{-1}. At given reaction times, samples were withdrawn from the reaction media and analysed for glucose and cellobiose by HPLC (Parajó *et al.*, 1995).

Micro-organism and fermentation assays

Freeze-dried broths of *X. dendrorhous* ATCC 24228 were proliferated in a medium containing 10 g glucose litre^{-1}, 3 g yeast extract litre^{-1}, 3 g malt extract litre^{-1} and 5 g peptone litre^{-1}, and transferred to plates containing the same medium supplemented with 20 g agar litre^{-1}. In selected experiments, enzymatic hydrolysates, or commercial sugar solutions, were supplemented with 0.1 g KNO_3 litre^{-1}. After inoculation with *X. dendrorhous* cells, fermentations were carried out in a batch fermenter (Biostat B, Braun Biotech). The experimental conditions for fermentation were: agitation speed controlled to provide 40% oxygen saturation, pH 6 and temperature 22°C. At given fermentation times, samples were withdrawn from the fermenter and centrifuged. Glucose and cellobiose were determined in supernatants by the same HPLC method cited above, and the pellets were washed twice with sterile water and used to measure both biomass (as dry weight) and carotenoids using separate aliquots. Carotenoids were extracted from cells by disruption with dimethylsulphoxide (DMSO; Sedmak *et al.*, 1990), transferred to hexane (Calo *et al.*, 1995) and analysed by the HPLC-DAD method reported elsewhere (Parajó *et al.*, 1997*c*).

RESULTS AND DISCUSSION

Chemical processing of wood and enzymatic hydrolysis

Native lignocellulosic materials are poor substrates for enzymatic hydrolysis, owing to several factors, including the presence of lignin and hemicelluloses (that hinder the access of enzymes to the heterocyclic ether bonds of cellulose) and the crystalline structure of cellulose

(Blanch and Wilke, 1983). Because of that, the raw materials have to be subjected to physical and/or chemical pretreatments before enzymatic hydrolysis. Alkaline treatment of *Eucalyptus* wood is an easy way to improve both the kinetics and the yields of the enzymic saccharification (Parajó *et al.*, 1992). Under selected operational conditions (10% NaOH, 130°C, 10 g alkaline solution g^{-1} dry wood, 2 h), several beneficial effects are provoked by the alkaline treatments, including partial delignification, hemicellulose hydrolysis and structural alteration of cellulose. These modifications of substrates enhance their susceptibility towards enzymatic hydrolysis. Additional data on the kinetics and yields of the enzymatic hydrolysis of pretreated wood with cellulases or cellulase–cellobiase mixtures are available in previous works (Parajó *et al.*, 1992, 1997*d*). As can be seen from Fig. 1 (that shows data on the alkaline treatment), the enzymatic saccharification of solid residues led to solutions containing glucose or glucose and cellobiose, depending on the formulation of the enzymatic complex. In a further step, hydrolysates were supplemented with nutrients or nutrients plus an inorganic nitrogen source (KNO_3) to give suitable proliferation media.

Fermentation assays

The four types of fermentation media made from hydrolysates are referred to as: HG (hydrolysates containing glucose as the sole carbon source and no inorganic nitrogen source); HGN (hydrolysates containing glucose as the sole carbon source and an inorganic nitrogen source); HGC (hydrolysates containing glucose and cellobiose as carbon sources and no inorganic nitrogen source) and HGCN (hydrolysates containing glucose and cellobiose as carbon sources with an inorganic nitrogen source). For comparative purposes, four synthetic media were prepared from commercial sugars with the same composition as the above-mentioned ones. The nomenclature used for these media (SG, SGN, SGC and SGCN) is similar to that used for media made from hydrolysates, the only difference being the change of the first character (S instead H, i.e. synthetic media instead of hydrolysate media). This experimental plan allows a direct comparison of hydrolysate and synthetic media, as well as evaluation of the effects caused by the presence of cellobiose or KNO_3. The presence of this last compound has been reported to support high pigmentation of mutant *Phaffia* strains (Fang and Cheng, 1993), whereas enhanced carotenogenesis in xylose-based media containing inorganic nitrogen sources has been recently reported (Parajó *et al.*, 1997*a*).

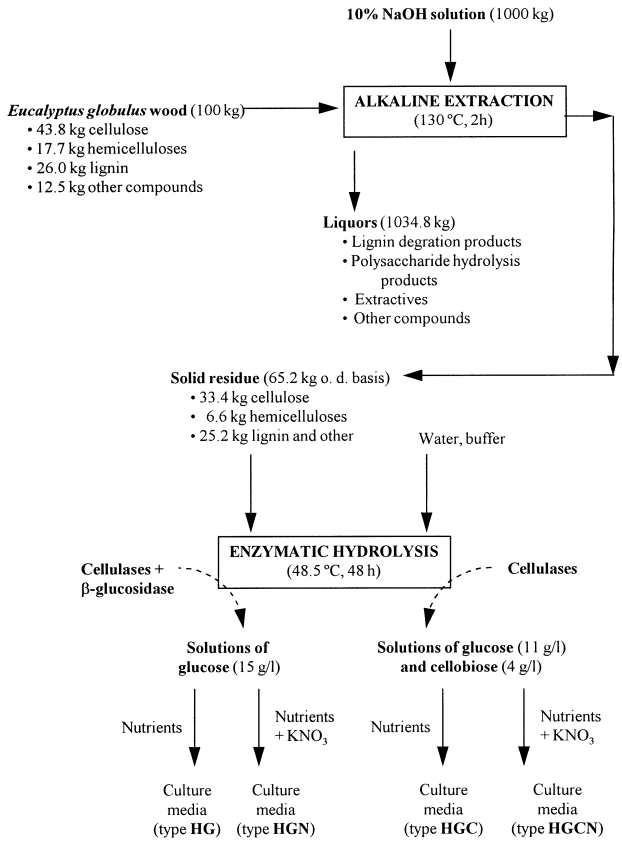

Fig. 1. Scheme of wood processing for making culture media.

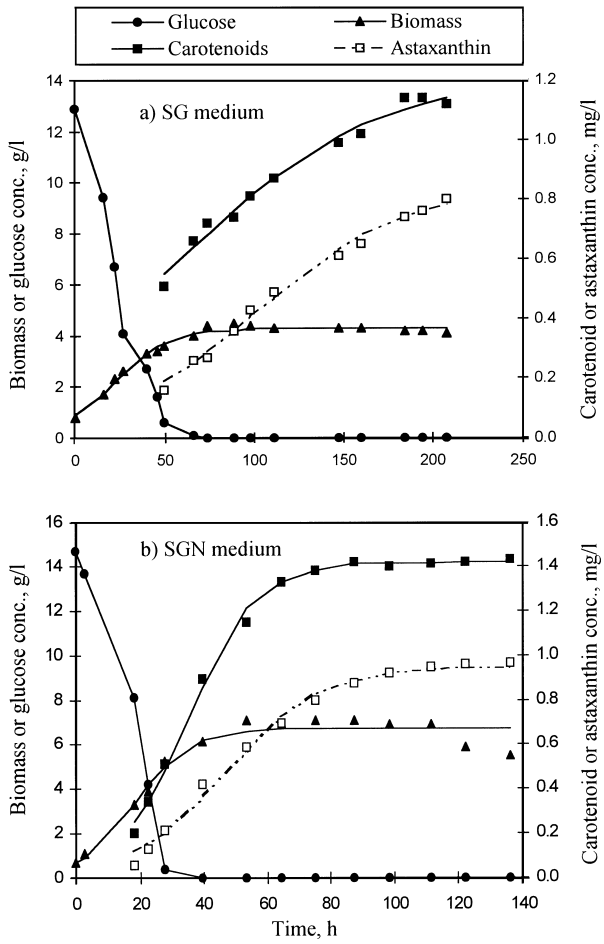

Fig. 2. Dynamics of fermentation carried out in: (a) synthetic culture medium containing glucose (medium type SG); (b) synthetic culture medium containing glucose and KNO_3 (medium type SGN).

Figures 2–5 show the experimental results found in fermentation experiments carried out with the media assayed. In these figures, the lines drawn for biomass, total carotenoids and astaxanthin correspond to the fitting of experimental data to the model proposed by Moraine and Rogovin (1966). A comparison between experimental and calculated results shows that the model of Moraine and Rogovin provided a satisfactory interpretation of the time courses of biomass, carotenoids and astaxanthin concentrations.

A qualitative evaluation of Figs 2–5 shows that all the culture media were suitable for the proliferation of the yeast. In media made from hydrolysates, glucose was depleted in 20–25 h, whereas substrate was consumed with slower kinetics in synthetic media. In this last case, the presence of KNO_3 improved the rate of glucose consumption. Longer fermentation times (about 70 h) were necessary for cellobiose consumption in media SGC, SGCN and HGC.

Biomass yields in the range 0.334–0.472 g biomass g^{-1} consumed substrate, were found for synthetic media. The corresponding values obtained for HG or HGN media (0.461 or 0.464 g g^{-1}) lie in the upper part of this range, whereas cellobiose-containing, hydrolysate media (type HGC or HGCN) led to higher biomass yields (within the range 0.54–0.59 g g^{-1}). The slopes of the curves of biomass concentration/time showed that, for a given type of supplementation, hydrolysate-based media supported higher rates of biomass generation than synthetic media, a fact that could be related to the presence of denatured enzymes or byproducts from enzymatic hydrolysis that could also be utilized by micro-organisms.

The fast dynamics of carotenoid generation in hydrolysate media is a remarkable finding. The maximum carotenoid concentrations were reached after 40–70 h in media made from hydrolysates, in comparison with 90–200 h in synthetic media. Because of this, the mean volumetric rate of carotenoid production during the exponential phase of growth is 5–6 times higher in the case of hydrolysate-based media. Particularly, cellobiose-containing, hydrolysate media (with or without KNO_3) led to significant improvements in carotenoid production in comparison with both synthetic media and the results reported in a previous work (Parajó *et al.*, 1997*c*), even if the results of this last study and the actual ones are difficult to compare owing to the different fermentation technology employed (shaken flasks

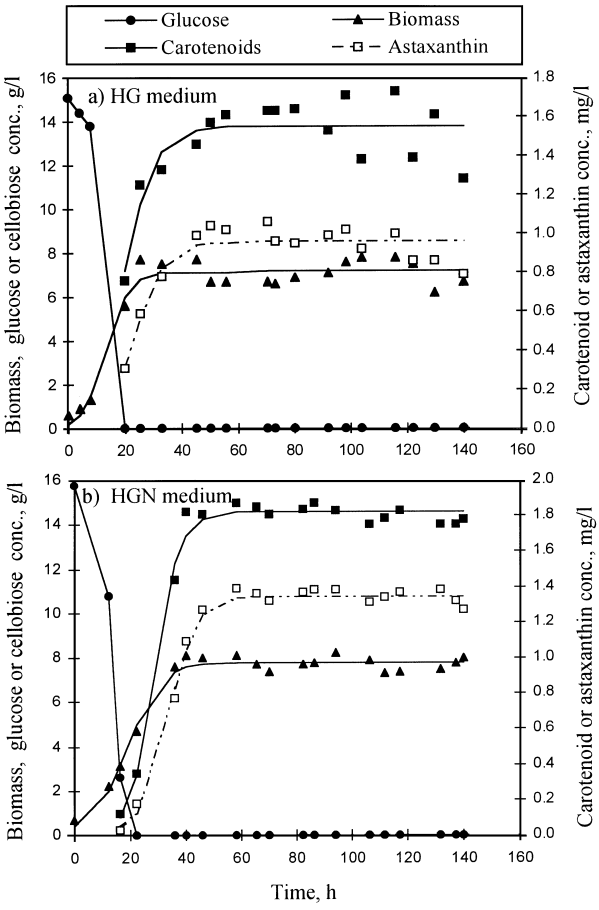

Fig. 3. Dynamics of fermentation carried out in: (a) medium made from hydrolysates containing glucose (medium type HG); (b) medium made from hydrolysates containing glucose and KNO_3 (medium type HGN).

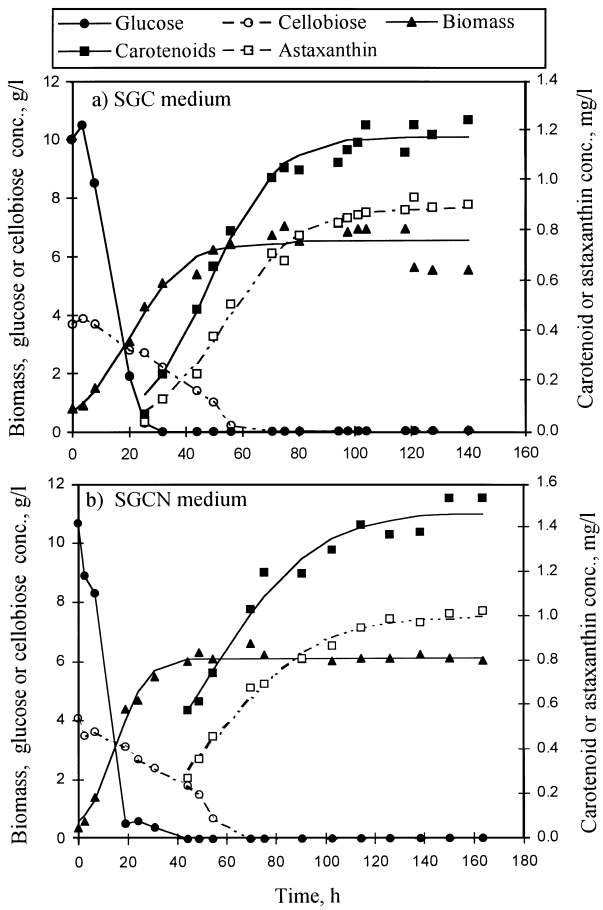

Fig. 4. Dynamics of fermentation carried out in: (a) synthetic culture medium containing glucose and cellobiose (medium type SGC); (b) synthetic culture medium containing glucose, cellobiose and KNO_3 (medium type SGCN).

and stirred bioreactor, respectively). Little beneficial effect (about 3% increase in the maximum carotenoid concentration predicted by the mathematical model) was associated with the presence of cellobiose in both synthetic and hydrolysate-based media. However, the presence of cellobiose significantly affected the carotenoid profile, increasing the astaxanthin percentage of total hydrolysates (from 61.7% in media without cellobiose up to 68.5% in media containing this disaccharide). Other carotenoids present in cells (up to 9.5% of their total amount) were 3-hydroxy-3′,4′-didehydro-β-ψ-carotene (HDC) and 3-hydroxyechinenone. Minor amounts of lycopene, echinenone and canthaxanthin (within the range 1–5% of total carotenoids) were also detected. On the other hand, significant increases in total carotenoid concentration were caused by the inorganic nitrogen source (24.5% in synthetic media or 17% in hydrolysate media). Accumulative effects derived from the presence of cellobiose and KNO_3 were observed, leading to a maximum total concentration of 2.05 mg total carotenoids litre^{-1} in media HGCN, 81.5% of this amount corresponding to astaxanthin.

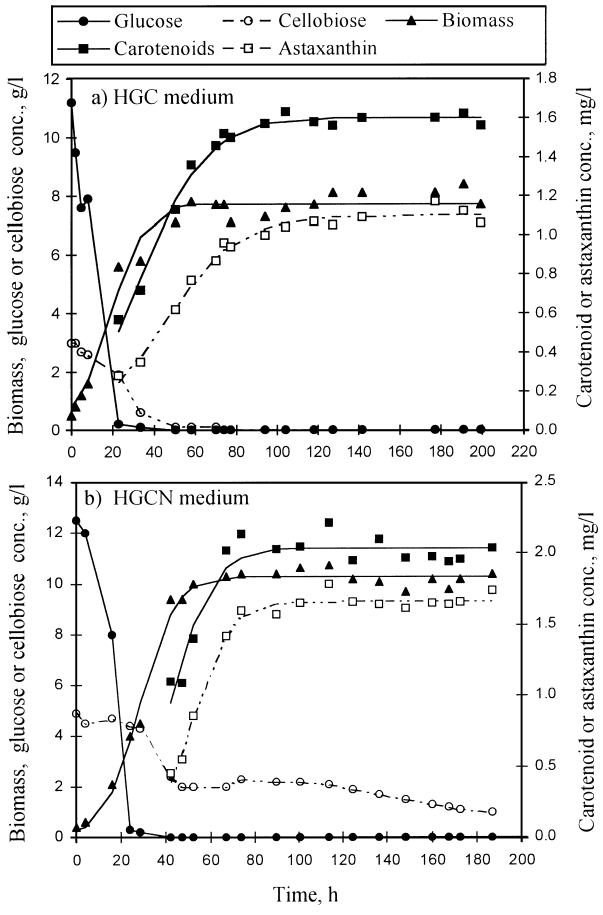

Fig. 5. Dynamics of fermentation carried out in: (a) medium made from hydrolysates containing glucose and cellobiose (medium type HGC); (b) medium made from hydrolysates containing glucose, cellobiose and KNO_3 (medium type HGCN).

CONCLUSION

Alkali-processed wood can be treated with a β-glucosidase-deficient cellulase complex (with or without additional β-glucosidase) in order to obtain solutions containing either glucose and cellobiose or glucose, both of them suitable for formulating culture media allowing the proliferation of red yeasts. Supplementation of hydrolysates with reduced concentrations of an inorganic nitrogen source (0.1 g KNO_3 litre^{-1}) resulted in improved cell pigmentation. The presence of cellobiose slightly improved the overall carotenoid concentration and directed carotenogenesis towards the synthesis of astaxanthin, whereas improved pigmentation was observed in media supplemented with KNO_3. In the best case assayed (hydrolysate-based media containing cellobiose and KNO_3), carotenoid concentrations above 2 mg litre^{-1} (maximum experimental concentration, 2.14 mg litre^{-1} after 74 h) were reached, with high astaxanthin proportion (81.5%). These results confirm the validity of the experimental procedure followed for improving the biotechnological production of carotenoids.

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