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Effect of the nutrient solution in the microbial production of citric acid from sugarcane bagasse and vinasse

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ABSTRACT

The process known as "solid-state cultivation" (SSC) refers to microbial growth on solid supports under limited water conditions. Citric acid is a bioproduct with several industrial applications, and its production is traditionally microbial. The present work evaluated the production of citric acid by microbial consortium of *Aspergillus niger* and *Trichoderma reesei* in sugarcane bagasse with vinasse and ethanol as inducer of the metabolic production of this organic acid. The results indicate that the highest yields of glucose in citric acid, productivity and specific production rate for the conditions having vinasse and vinasse/ethanol as nutrient solution. In addition, the kinetic parameters for the citric acid and glucose profiles indicate that only vinasse as a nutrient solution, has a maximum citric acid concentration of 734 mg L^{-1} in 48 h.

1. Introduction

Citric acid is a carboxylic acid commonly found in the metabolism of plants, animals and microorganisms (Dhillon et al., 2011). Because it is one of the most important organic acids, it is widely used in the food, beverage, chemical and metallurgical industries (Zhang et al., 2017). Due to the wide application of citric acid, its microbial production continues to be of interest for extensive study (Angumeenal and Venkappayya, 2013). Moreover, the large demand of citric acid indicates the need to find alternatives for its efficient production either using low-cost substrates or by improving the potency of the microorganisms (Chetan et al., 2018). In Brazil, the demand of this product in the national market has been supply by imports, and, in addition, there is a constant increase of about 4% in citric acid consumption each year, which demonstrates a need to find alternatives for its manufacture (Soccol et al., 2006).

A large number of microorganisms including bacteria, fungi and yeasts has been use for the production of citric acid. The most widely used industrial production process is submerged cultivation mainly *Aspergillus niger* from starch or sucrose (Chen and Nielsen, 2016; Dezam et al., 2017). However, in the last years there has been a several reports about the use of many substrates as lignocellulosic agro-industrial wastes (Shojaosadati and Babaeipour, 2002; Kumar et al., 2003; Khosravi- Darani and Zoghi, 2008; Dhillon et al., 2011; Angumeenal and Venkappayya, 2013; Dhillon et al., 2013). In this sense, the solid-state cultivation emerges as an alternative to submerged microbial processes due to increase the demand for citric acid production (Dezam et al., 2017).

Solid-state cultivation (SSC) can be define as the growth of microorganisms on solid supports under conditions near to the absence of free water. In general, the solid medium acts as a physical carrier providing nutrients containing water that meets the requirements for microbial growth. Thus, costs with SSC may be lower than submerged processes, even though agro industrial by-products are often used as solid support (Barrington et al., 2009; Thomas et al., 2013). Thus, SSC is a very promising technique for use of alternative substrates, leading to low-cost and ecofriendly process (Vandenberghe et al., 2018).

Mixed cultures of microorganisms can complement the metabolic capacities of microorganisms, generating secretion products with more adequate profiles, increasing the bioconversion capacity of substrates (Gutiérrez-Correa and Villena, 2017). In addition, the advantages of using mixed cultures may be more evident in the SSC, since colonization, penetration and degradation of the solid-state substrate. It tends to be intensified by symbiotic association, where each species may have its own niche for growth and degradation of the substrate due to its own matrix of hydrolytic enzymes, which may act with other synergistically (Gutiérrez-Correa and Tengerdy, 1997).

Brazil is the largest producer of sugarcane in the world, accounting for around 25% of total world production (Sindhu et al., 2016). Sugarcane bagasse with 46–52% of moisture is composed (dry basis) around 43–52% of fibers (including 50% cellulose, 25% hemicellulose and 25% of lignin), 30% of sugars and 2.4% of ash (Pandey et al., 2000;

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Mazutti et al., 2006; Mussatto et al., 2012). Thus, sugarcane bagasse presents characteristics of solid support for SSC, being able to serve as a source of carbon and/or nutrient for the generation of a variety of add-value compounds. On the other hand, vinasse is the main wastewater in the from ethanol fermentation-distillation due to the high volume generated and the high organic matter (Navarro et al., 2000; Madejón et al., 2001). For each liter of ethanol produced, more than 10 L of vinasse can be generated, totaling approximately 300 billion liters per year of this wastewater in Brazil (CONAB, 2013). Thus, vinasse could be used as a nutrient solution for the SSC by impregnating the sugarcane bagasse particles, minimizing the cost of production and taking advantage of two by-products generated in the same industrial platform.

In this context, the aim of this research was to evaluate the citric acid production by fungal consortium (*Aspergillus niger* and *Trichoderma reesei*) in SSC from sugarcane bagasse with different impregnating solutions.

2. Material and methods

2.1. Inoculum

Aspergillus niger CCT 4355 and *Trichoderma reesei* were kept in standard PDA medium in the Laboratory of Applied Microbiology (Center of Agricultural Sciences CCA/UFSCar, Araras/SP). Previously to each experiment, inocula were grow at least seven days in Erlenmeyer's Flasks in liquid medium with 20% sucrose, 0.25% ammonium nitrate, 1% potassium phosphate (KH_2PO_4), 0.025% magnesium sulfate and 0.004%. Inoculum preparation followed the procedures according previous studies from research group (Oliveira et al., 2012; Bastos et al., 2015; França, 2016).

2.2. Sugarcane bagasse and vinasse

Sugarcane bagasse and vinasse used in the experiments were collected in a sugarcane processing industry located in the Araras/SP. Particle size of the sugarcane bagasse was choose between 0.59 and 1.17 mm of mean diameter with a set of TYLER sieves, with subsequent sterilization in polypropylene bags.

Vinasse used as impregnating solution of the sugarcane bagasse particles was sterilized at 121 °C for 20 min in autoclave, being characterized in terms of pH by potentiometry, glucose content by enzymatic glucose oxidase-peroxidase with kit LABORLAB®, carbon and total nitrogen in SHIMADZU® TOC-LCPN Analyzer.

2.3. SSC exploratory tests from vinasse and ethanol as impregnating solution of the sugarcane bagasse particles

SSC were performed in packed-bed columns (Figs. 1 and 2) with

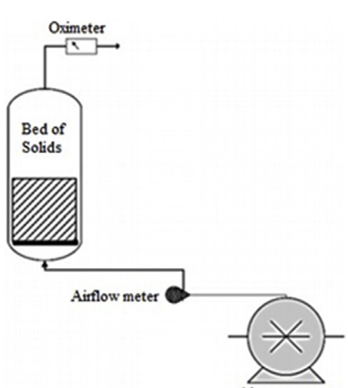

Fig. 1. SSC scheme with the sugarcane bagasse composing of the packed-bed (adapted from Bastos et al., 2015).

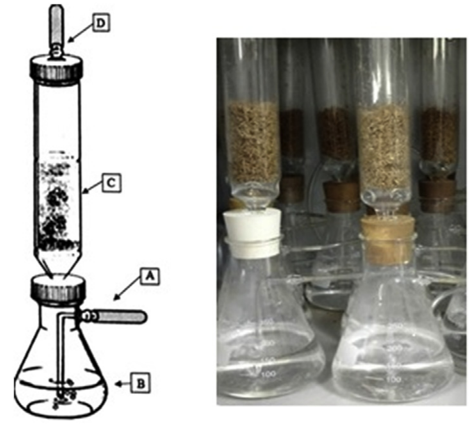

Fig. 2. Detail of the one packed-bed column bioreactor used in SSC (adapted from Motta and Santana, 2014). A: air inlet for humidification; B: distilled water for humidification; C: solid packed-bed; D: air outlet of the column.

30 mm in diameter and 200 mm of bed height, filled to 60 mm with the selected and sterilized particles of sugarcane bagasse (solid support) with initial moisture of 80% (impregnating solution and inoculum suspension). Experiments were set up at 30 °C with continuous airflow. Four different moistening solutions added together with the spore suspension of *Aspergillus niger* and *Trichoderma reesei* consortium: 1% sucrose (mass/volume), 1% sucrose with ethanol at 4% (mass/volume), only vinasse and vinasse with 4% ethanol. At this step, the samples were incubate for 4 days.

Solid moisture of the support was determined by dry mass. Fungal extracts were obtained in each treatment with the addition of deionized water in 1:15 ratio (solid-solvent), shaking for 45 min at 100 rpm and 28 °C, with subsequent addition of acetone, according methodology proposed by Khosravi-darani and Zoghi (2008) and adapted by Bastos et al. (2015).

Fungal extract was characterized in terms of pH by potentiometry, the glucose content by the enzymatic glucose oxidase - peroxidase (LABORLAB®), citric acid by colorimetric reaction with pyridine and acetic anhydride using commercial kit IN VITRO® and Dionex® Ultimate 3000 HPLC (Pereira et al., 2010) and carbon/nitrogen total by SHIMADZU® TOC-LCPN.

Maximum productivity in terms of citric acid (Prod) and the specific rates of production (μp) were calculated considering the elemental general formula for fungi ($CH_{1,72}O_{0,55}N_{0,17}$) as reported by Nielsen et al. (2003), using the total nitrogen concentration.

Citric acid yield obtained in the SSC ($Y_{P/S}$) tests can be estimated in order to compare with the stoichiometric, i.e., 0.8 mol of carbon produced (citric acid) per mole of carbon of the substrate consumed (Papagianni, 2007).

2.4. Profiles of citric acid and glucose under the previously selected SSC conditions

SSC were set up as described in the previous section, but with monitoring every 24 h for 144 total hours, in order to evaluate both the production of citric acid and the release of glucose in the medium. In these cases, sugarcane bagasse was impregnated with only vinasse and vinasse and ethanol at 4% (mass/volume) and the consortium of *Aspergillus niger* and *Trichoderma reesei*. The assays were done in triplicate and the extraction of the extract, as well as the monitoring analyzes, were performed according to the previous item during the experimental period.

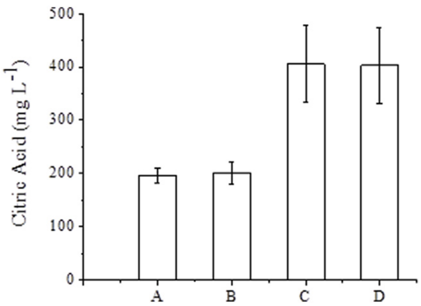

Fig. 3. Citric acid contents in the fungal extract for 4 days of SSC in sugarcane bagasse impregnated with (A) sucrose, (B) sucrose and ethanol, (C) vinasse and (D) vinasse and ethanol.

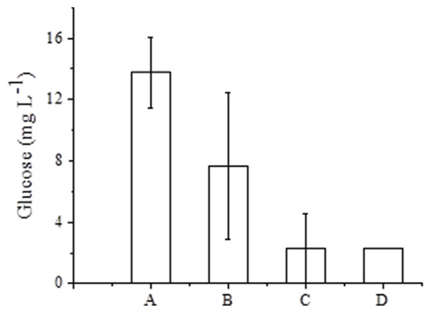

Fig. 4. Glucose contents in the fungal extract for 4 days of SSC in sugarcane bagasse impregnated with (A) sucrose, (B) sucrose and ethanol, (C) vinasse and (D) vinasse and ethanol.

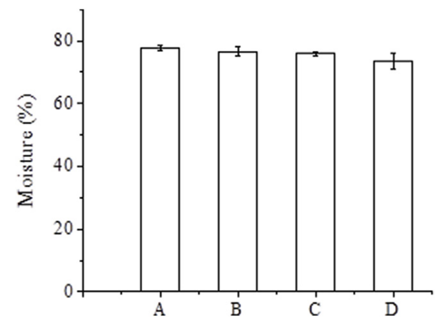

Fig. 5. Profiles solid moisture for 4 days-SSC in sugarcane bagasse impregnated with: (A) sucrose, (B) sucrose and ethanol, (C) vinasse and (D) vinasse and ethanol.

3. Results and discussion

3.1. SSC exploratory tests from vinasse and ethanol as impregnating solution of the sugarcane bagasse particles

SSC fixed the batch time in 4 days, pre-selected time by previous researches (França, 2016), using vinasse and ethanol as stimulants of the microbial production of citric acid. According to Fig. 3, it was possible to observe higher citric acid contents using vinasse and vinasse/ethanol as impregnant solution, 405 and 402 $mg\,L^{-1}$, respectively. As all other conditions were keep constant, the difference in citric acid results was due to impregnating solutions. Pal and Khanum (2010) indicate that nutrient solutions affect performance of SSC. Dhillon et al. (2013) reported that ethanol and/or methanol induce the production of citric acid, however, may inhibit sporulation depending of concentration. According to the our results, the addition of vinasse and ethanol was more beneficial for citric acid production than inhibitory. It should be noted that the vinasse could originally presents a minimum content of ethanol due to the conduction of alcohol distillation (around 0.5% mass/volume), which may have induced this production. The use of inferior alcohols as a stimulant for the production of organic acids by fungi may be linked to changes in their morphology. Nadeem et al. (2010) obtained slightly higher yields using alcohols as stimulants for the accumulation of citric acid, mainly with methanol. Considering that this alcohol is difficult to handle and with prospects the process escalation, it was suggested the use of ethanol, with reduced cost and abundant in Brazil. Moreover, vinasse presents organic matter mainly in the colloidal form and several salts, which can contribute to the production of citric acid. According to Papagianni (2007), *Aspergillus niger* requires concentrations of various trace metals for the production of citric acid and growth, such as zinc, manganese, iron and copper. Vinasse used in the tests presented a favorable C/N ratio of around 20 (total organic carbon 1036 $mg\,L^{-1}$ and total nitrogen 50.27 $mg\,L^{-1}$), zinc contents (0.69 $mg\,L^{-1}$), copper (0.035 $mg\,L^{-1}$), iron (14.5 $mg\,L^{-1}$) and manganese (3.11 $mg\,L^{-1}$). Thus, the composition of the vinasse certainly influenced positively the citric acid production in comparison to the conditions with sucrose, where the productivity was much lower, directing the substrate for cell growth.

In this sense, Fig. 4 shows the residual glucose concentrations during SSC under these conditions. According to the results, for vinasse conditions, there was also the lowest concentration of residual glucose. Part of the organic carbon in the vinasse is available in the form of glucose, resulting from pre-treatment and prior sterilization of the experiments (De Mattos and Bastos, 2016). Therefore, in the case of sucrose, the microbial hydrolysis in the two hexoses must occur for the sequence of the aerobic metabolism via glucose, Krebs Cycle and respiratory chain. According to Angumeenal and Venkappayya (2013), the quality and quantity of carbon sources influence the microbial metabolic activity for citric acid production, with glucose as a principal carbon source (see Fig. 5).

Table 1
Observed yields of glucose in citric acid ($Y_{P/S}$), yields (P_{rod}) and specific rates of citric acid production (μ_P) for sugarcane bagasse cultures with fungal consortium.

Impregnating solution	$Y_{P/S}$ ($mg_{citric\ acid}\ mg_{glucose}^{-1}$)	P_{rod} ($mg_{citric\ acid}\ L^{-1}\ h^{-1}$)	μ_P ($mg_{citric\ acid}\ g_{biomassa}^{-1}\ h^{-1}$)
Vinasse	1,31	4,22	8,97
Vinasse and ethanol	1,29	4,19	8,91
Sucrose	0,66	2,05	4,36
Sucrose and ethanol	0,67	2,79	5,94

Such results can be discuss from the kinetic data presented in Table 1. The higher yields, productivity and specific citric acid production rates (in terms of biomass) for vinasse conditions indicating the viability of the SSC under these conditions. As the stoichiometric yield in citric acid is reported as 0.8 mol per mole of substrate, this parameter cannot be expressed in this in percentage terms, according reported in literature (Papagianni, 2007; Nadeem et al., 2010). In this case, 1.31 and 1.29 $mg_{citric\ acid}\ mg_{glucose}^{-1}$ (values higher than 100%) may be the result of the glucose release from hydrolysis of the structural polysaccharides, difficult to detect during the process, increasing the amount of total substrate. Thus, instead of the actual amount of glucose converted to citric acid, one has the observed yields in terms of mass.

Oliveira et al. (2012), in a pioneering research combining sugarcane bagasse and vinasse, obtained productivities around 1.45 g per gram of solid medium per day in terms of total acids with only *Aspergillus niger*. Kumar et al. (2003) reported yields around 30% of the stoichiometric maximum in 9 days with bagasse impregnated with nutrient solution and addition of methanol. Bastos et al. (2015) evaluated the production of citric acid by *Aspergillus niger* from sugarcane bagasse and vinasse in packed-bed column with different bed heights. These authors verified higher production in 3 days (1.75 g per 100 g of solid medium), with higher glucose release at 120 mm of bed height. Vandenberghe et al.

(2000) reported yields of 88 g of citric acid per kg of dry matter for cassava bagasse, 48.7 g kg $^{-1}$ for sugarcane bagasse and 12.7 g kg^{-1} using bark coffee. As reported by Bastos et al. (2014), a production of 27.1 g per kg of sugarcane bagasse was obtain at the maximum concentration point of citric acid at 200 mm bed height with aeration of 3 L min $^{-1}$. Anyway, the use of vinasse as a nutrient solution impregnating sugarcane bagasse is promising and interesting since it adds the use of two by-products from sugarcane processing.

SSC studies involving the initial solid moisture selection, fundamental in these microbial growth, neglects the transient regime, i.e., the tendency of variation of this parameter during the time depending. The moisture in the solid medium tends to rise as respiration generates considerable amounts of metabolic water (6 mol of H_2O for each mole of glucose consumed). Therefore, if the bioreactor cannot disperse this water to the gas phase, there is a water accumulation in the bed, leading to limitations mainly in the oxygen transfer. This water drag in the columns depends of the airflow, gas humidity and bed height. Bastos et al. (2015) maintained the solid moisture almost constant from the selection of bed height and airflow. This maintains the original characteristics of the SSC, i.e., conditions close to the absence of free water, without non-limiting oxygen effects or considerable variation in the porosity or thickness of the liquid film deposited on the surface of the particles. Kumar et al. (2003) report maximum sugars consumption by *Aspergillus niger* at SSC from fruit residues at a moisture content of 70%. Above this level, both the production of citric acid and the consumption of sugars decrease, which could be explain by the authors due to the reduction of the porosity of the bed of particles, with consequent limitation of the transfer of heat and mass.

According to Lekanda and Pérez-Correa (2004), the water balance can reach the permanent regime when the rate of metabolic water generation is equivalent to the consumption of solid support, with loss of mass.

The results presented in Fig. 6 indicate that, for all experimental conditions, solid moisture remained at 4 days discreetly below the initial value of 80%, suggesting adequate conditions of oxygen transfer and water balance for SSC conditions.

3.2. Profiles of citric acid and glucose under the previously selected SSC conditions

Considering the data from Table 1, vinasse and vinasse/ethanol as nutrient solution showed the best results of yield and productivity. Thus, Fig. 7 shows the citric acid and glucose profile using only vinasse as an impregnate solution. Citric acid production presented a satisfactory level in the first 72 h of SSC, with maximum in 48 h (734.52 mg L^{-1}). Glucose profiles indicate an increase in glucose content, followed by a considerable consumption phase up to 48 h, which corresponds to the period of greatest production of citric acid. According to discuss previously, the maintenance of solid moisture for 144 h of SSC demonstrates a physical stability of the particle bed, without major limitations of heat transfer and mass. As was the case for the previous tests, the solid medium moisture remained practically constant, indicating a physical stability of the particle bed and non-

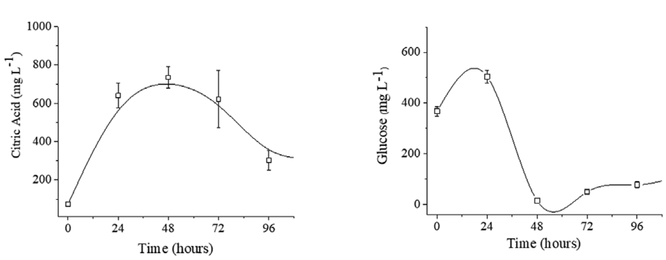

Fig. 6. Profiles of citric acid in the fungal extract for SSC in sugarcane bagasse impregnated with vinasse.

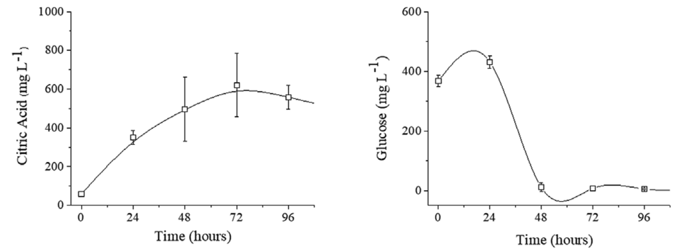

Fig. 7. Citric acid and glucose profiles in the fungal extract for SSC in sugarcane bagasse impregnated with vinasse and ethanol.

limiting oxygen and heat transfer.

According to Haq et al. (2003), the use of lower alcohols, such as ethanol and/or methanol tends to increase the yield of citric acid due to changes in the permeability of the cell membrane. Fig. 7 present the highest amount of citric acid occurred in 72 h of SSC, (about 620 mg L^{-1}), using vinasse and ethanol as impregnating solution of the sugarcane bagasse particles.

Glucose profiles indicate a higher consumption of glucose by the microorganisms in 72 h of SSC, concomitant to the greater production of citric acid. However, after this time, glucose levels remained lower, maintaining a steady state. According to Oliveira et al. (2012), this is common for SSC where the solid support contains the carbon source, since there is glucose consumption and release via enzymatic hydrolysis of the lignocellulosic structure by fungi.

Table 2 summarizes the results in terms of the kinetic parameters. Results indicate that the selection of the batch time is fundamental for these SSC. Maximum values obtained in the ideal batch time were higher than those found in Tables 1 and i.e., yield (1.88 mg $_{\text{citric acid}}$ mg $_{\text{glucose}}{}^{-1}$), productivity (13.77 mg $_{\text{citric acid}}$ L^{-1} h $^{-1}$) and specific rate of citric acid production (29.3 mg $_{\text{citric acid}}$ $g_{\text{biomassa}}{}^{-1}$ h $^{-1}$) with only vinasse as a nutrient solution.

The yield in terms of mass of citric acid produced by mass of glucose consumed is higher than the results presented in the literature. Kumar et al. (2003) working with *Aspergillus niger* DS 1 from sugarcane bagasse and sucrose and molasses based medium obtained in optimized conditions (73% initial moisture, 4% methanol as inductor in particles of 1,2 to 1.6 mm and 31.8 g of sugar per 100 g of solid medium) yields at around 0.7 g g $^{-1}$ at 9 days. As already mentioned, yields greater than 1 g g^{-1} indicate that not only the carbon source of the nutrient solution is used, but also the structural polysaccharides of the solid medium. The results of maximum productivity are lower than the data presented by Nadeem et al. (2010) (256 mg L^{-1} h^{-1}). However, it should be noted that these authors used *Aspergillus niger* M-101 and methanol as an inducer in concentrations of 0.5–2% (volume by volume) in stirred flasks with an optimum medium based on beet molasses. On the other hand, the specific production rate is higher than that reported by these authors (6 mg g $^{-1}$ h $^{-1}$), which indicates an adequate adaptation of the inoculum to the solid medium containing sugarcane bagasse and vinasse, i.e., the higher yield of citric acid is obtained by biomass. In this context, the results in Table 2 are important and promising, even as it is a biotechnological production from two agro industrial by-products.

4. Conclusions

According experimental results, it is possible to conclude that the use of vinasse and ethanol as impregnating solution of the sugarcane bagasse particles is efficient for the production of citric acid, with ideal batch time to obtain higher production for solid-state cultivation with consortium of *Aspergillus niger* and *Trichoderma reesei*.

Conflicts of interest

The authors have no financial conflicts of interest to declare.

Table 2
Maximum observed yields of glucose in citric acid ($Y_{P/S}$), maximum yields ($Prod_{max}$) at the respective times and specific rates of citric acid production (μ_P) for sugarcane bagasse cultivations fungal consortium with vinasse and vinasse/ethanol in 144 h.

Impregnating solution	$Y_{P/S\ máx}$ ($mg_{\ citric\ acid}\ mg_{\ glucose}^{\ -1}$)	$P_{rod\ máx}$ ($mg_{\ citric\ acid}\ L^{-1}\ h^{-1}$)	μ_P ($mg_{\ citric\ acid}\ g_{\ biomassa}^{\ -1}\ h^{-1}$)
Vinasse	1.88	13.77	29.30
Vinasse and ethanol	1.56	7.79	16.58

Acknowledgments

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