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Citric acid production and citrate synthase genes in distinct strains of *Aspergillus niger*

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Citric acid is an important organic acid, multifunctional with a wide array of uses. The objectives of this study were the isolation and selection strains of the genus *Aspergillus*, investigating the solubilization of phosphate of these isolates, verifying the expression rate of genes involved in the identification of isolates, and efficiency of citrate synthase in the citric acid production. To verify the mechanisms of the microorganisms in solubilizing phosphate into the medium in the citric acid production, Araxá rock phosphate was utilized. Further, citric acid was evaluated in two culture media, namely BD and SA. The amplification by the polymerase chain reaction was done by using primer ITS region and citrate synthase (P1/P2). The isolates of *Aspergillus niger* were efficient to solubilize phosphate. The isolate *A. niger* 00118 stood out in the solubilization with an increase as high as five times the amount of soluble phosphorus when compared with the control treatment. The results of citric acid production highlighted better influence conditions of the culture medium Sabouraud (SA) on its production. The P1/P2 primer was sensitive in distinguishing the isolates *A. niger* 00116, *A. niger* 00104, *A. niger* 00098 and *A. niger* 00118 in the analysis of citrate synthase enzyme genes.

Key words: Biotechnology, enzyme, strains.

INTRODUCTION

Citric acid is the leading constituent of citrus fruits, currently one of the most important organic acids produced by microbial pathway. Because of its characteristics, it is widely utilized in food industry (acidulants, flavorings, antioxidants), in the pharmaceutical industry (buffering, scavenger, chelating) and others. Its importance is due to the characteristics (such as palatability, low toxicity) that it is easily assimilated by

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the organism. With the use of microbiological process technique, it was possible to obtain acid by reducing the obtaining cost and greatly increasing the use in industrial scale.

The metabolic pathway established involved in the citric acid biosynthesis includes citrate synthase and this enzyme is most time, considered fundamental in the citric acid cycle, maybe as the first step of the cycle. The enzyme catalyzes the reaction of condensation of acetate and acetyl coenzyme A with an oxaloacetate molecule to form citrate (Papagianni and Mattey, 2007).

As the accumulation of citric acid by *A. niger* is accompanied by the action and activation or disappearance and reduction in the activity of some Krebs cycle enzymes, the utilization of molecular techniques may be an alternative viable for the characterization and identification of the gene, which codifies for the citrate synthase in lactic acid production by *A. niger* and with the use of species-specific primers of the genus, it is possible to improve the detection techniques to the desirable extent of performing this operation directly in selected isolates, making the process faster.

From among the molecular techniques which stand out, those ground on the DNA sequences amplification by the polymerase chain reaction (PCR) are included. The polymerase chain reaction is a highly sensitive technique by means of which small amounts of unique DNA or RNA sequences may be enzymatically amplified till millions of copies of the target sequence may be obtained (Gandra et al., 2008).

A number of microorganisms, including both bacteria and fungi, possess the capacity of solubilizing inorganic phosphates by means of different mechanisms. The solubilization of inorganic phosphates takes place through the decrease of the pH value caused by the microbial production of organic or inorganic acids releasing soluble phosphate (Barroso and Nahas, 2005). In this context, the objectives of this study were isolation and selection of strains of the genus *Aspergillus*, examining the solubilization of phosphate of these isolates, verifying the level of expression of genes involved in the identification of isolates and efficiency of citrate synthase with the citric acid production aiming at the selection of superior isolates for industrial production.

MATERIALS AND METHODS

Microorganisms

By the direct plating method, samples of soil, fruits, grains and breads were utilized for selection of *A. niger* for further purification. The characterization and the identification of the species of the genus *Aspergillus* was conducted on the basis of the taxonomy of Pitt and Hocking (1997).

From these, 12 isolates of *A. niger* were selected (00098, 00100, 00102, 00104, 00106, 00107, 00108, 00114, 00116, 00118, 00119, 00124) with best performance as to the capacity of solubilizing phosphate and producing citric acid.

Efficient phosphate solubilization and acid citric production

To verify the mechanism of phosphate solubilization of the isolates, Araxá rock phosphate was utilized, which was added into dose of 3 g/100 ml into liquid culture medium GL (glucose, yeast extract). Aliquots of 1 ml of suspension of the fungi 10^8 CFU (cells ml^{-1}) were transferred, singly, into a 250 ml Erlenmeyer (flasks) containing 100 ml of liquid culture medium and 3 g of phosphate as a source of phosphorus (P). The culture medium plus the phosphate with no inoculum was established as controls (Control 1) and the culture medium (Control 2). The treatments were inoculated at 28°C under stirring of 190 rpm for eight days, three replications being utilized. To quantify the soluble phosphorus (P) (deletion, it was earlier), the colorimetric method of Murphy and Riley (1962) was used. The pH was also determined according to the Association of Official Agricultural Chemists (AOAC) technique (1992).

Citric acid production

Citric acid was determined in two sorts of culture media, Sabouraud (SA) and Potato Dextrose (BD). Components BD medium include potato extract (200 g/500 ml), dextrose (20 g) and distilled water (1000 ml). SA media components include casein hydrolyzate (5 g), peptic hydrolyzate of animal tissue (5 g), glucose (40 g) and distilled water (1000 ml). Aliquots of 1 ml of suspension at 10^8 CFU (cells ml^{-1}) of each of the 12 isolates of *A. niger* in the two culture media (BD and SA) were inoculated into Erlenmeyer flasks of 125 ml containing 50 ml of culture medium. The treatments were incubated at 28°C under stirring of 140 rpm for 7 days; three replications were utilized. After the incubation period, citric acid in the medium with the different isolates of *A. niger* was determined.

Statistical analysis

For the soluble phosphorus analysis, the experimental design utilized was completely randomized with 14 treatments and three replications. For citric acid, the experiment was also conducted according to a completely randomized experimental design, nevertheless, the treatments were arranged in conformity to a factorial scheme 12×2 with 12 isolates of *A. niger* and two culture media (BD and SA) amounting to 24 treatments. The media obtained were compared by the Tukey test at 5% of probability, utilizing the SISVAR program (Ferreira, 2011).

DNA extraction and amplification

For DNA extraction, the samples of the isolates were ground in liquid nitrogen and approximately 1 g of each samples/isolate was taken, and for each replication used, 0.04 g was weighted for DNA extraction. DNA extraction of the samples coming from the 12 isolates of *A. niger* was done by means of *Wizard*™ Genomic DNA Purification Kit (Promega, Madison, WI, USA).

For amplifications by the polymerase chain reaction (PCR), the pairs of primers of the sequences Internal Transcriber Spacer 1 and 4 (ITS 1 and ITS 4) (TCCGTAGGTGAACCT GCGG/ TCC TCCGCTTATTGATATGC) reported by Mirhendi et al. (2007) were utilized to amplify regions of the rDNA of the 12 isolates of *A. niger* and for analysis of the citrate synthase gene in the isolates, the pairs primer (GCGAATTCATGTCTACCGGCAAGGCCAAGTCC/GCCCCGGGT CATTT ACAGCTTAGCACC), reported by Kirimura et al. (1999) were utilized. The gel was stained with red gel and the PCR products were observed in an UV transilluminator.

Table 1. Efficient solubilization of phosphate Araxá by isolates of *Aspergillus niger* in liquid medium.

Strain	Soluble phosphorus (mg/kg)	pH
A. niger 00118	77.23± 0.81[a]	1.87± 0.01[a]
A. niger 00104	66.41± 0.65[b]	1.91± 0.02[a]
A. niger 00114	56.84± 0.72[c]	2.01± 0.01[b]
A. niger 00116	51.12± 0.69[d]	2.11± 0.04[c]
A. niger 00098	47.66± 0.54[e]	2.27±0.05[d]
A. niger 00119	42.04± 0.93[f]	2.33± 0.01[e]
A. niger 00107	35.24± 0.35[g]	2.41± 0.01[f]
A. niger 00108	33.94±0.65[h]	2.52±0.06[g]
A. niger 00124	28.54± 0.82[i]	2.97±0.01[i]
A. niger 00106	26.42± 0.63[j]	3.04± 0.02[i]
A. niger 00100	25.37± 1.52[l]	2.97± 0.01[h]
A. niger 00102	19.29±0.25[m]	3.21± 0.03[j]
Control 1	14.69±0.64[n]	6.85± 0.01[l]
Control 2	1.13± 0.84[o]	7.02± 0.01[m]

Means followed by the same small letter in the column do not differ from one another by the Tukey test at 5% of probability.

RESULTS AND DISCUSSION

Efficient solubilization of the phosphate

In liquid medium, all the 12 isolates of *A. niger* presented additions in the amount of soluble phosphorus with Araxá rock phosphate. The isolates reduced pH of the liquid medium in relation to the control with no inoculation. The results are shown in Table 1.

That solubilization of phosphate related with the decrease of pH demonstrates the possible solubilization mechanism and acid production. The study of Chuang et al. (2007) indicated the significant and positive correlation between phosphate and acidity. For solubilization to exist, there is need of acid production. That same mechanism was also related in the report of Vassilev et al. (2006), citric acid being considered as a strong solubilizing agent.

According to Barroso and Nahas (2005), increasing titrable acidity, decrease in the values of final pH occurs which correspond to the increase of the soluble phosphate content. In general, isolate *A. niger* 00118 stood out from the other treatments with the greatest amount of soluble phosphorus and lowest pH, increasing on the average about as many as 5 the amount of soluble phosphorus in the medium when compared with Control 1.

That result can be ascribed to the best adaptation of the isolate to the culture medium highlighting an increased capacity to produce acidifying metabolites for phosphate solubilization. That indicates that the solulibizing capacity differs among the different isolates and that one can be better than the other.

Citric acid production

As regards the citric acid production, variation among the isolates was found and that of *A. niger* 00118 was the most efficient attaining a concentration of 18.42 g/(100 g) of citric acid in SA medium and 8.62 g/(100 g) in BD medium; being superior to the production obtained among the isolates studied. The results are shown in (Table 2).

In the work of Soccol et al. (2006), the production of citric acid by submerged fermentation by utilizing strains of *A. niger* and control of concentration of sugar, metals and oxygen obtained concentrations of about 14.98 g/L of citric acid. Prado et al. (2005), with the fermentation in the solid state of cassava bagasse utilizing also *A. niger* obtained an accumulation of citric acid corresponding to 26.9 g/(100 g).

The production of citric acid was greater in the SA medium than in BD medium. The results presented highlighted the influence of the conditions of the medium on citric acid production. The fact can be explained by the difference of the sugar and protein concentration present in the SA medium as glucose and peptone, making greater energy for the isolates available with the best sources of carbon and nitrogen. This occurrence is in accordance with Soccol et al. (2006), who observed poor output of citric acid owing to the fact that the sugar concentration is low in the medium.

The pH values of the 2 media studied had a small difference, the lowest being for those of the BD medium and the highest values for the ones of the SA medium. The results do not implicate in the linking between pH a citric acid production. The results are shown in (Table 3).

Table 2. Citric acid production by isolates of *Aspergillus niger* in submerged cultivation.

Strain	Citric acid BD medium (g/100 g)	Citric acid SA medium (g/100 g)
A. niger 00098	7.81± 0.04Ba	12.34± 0.01Eb
A. niger 00100	1.97± 0.06La	7.56± 0.02Hb
A. niger 00102	2.02± 0.02La	7.15± 0.04Ib
A. niger 00104	7.69± 0.02Ca	12.83± 0.06Cb
A. niger 00106	3.06± 0.03Ia	10.15± 0.06Gb
A. niger 00107	4.72± 0.02Ga	11.25± 0.08Fb
A. niger 00108	4.02± 0.06Ha	11.21± 0.03Fb
A. niger 00114	6.47± 0.08Da	13.63± 0.04Bb
A. niger 00116	6.33± 0.04Ea	12.82± 0.08Cb
A. niger 00118	8.62± 0.06Aa	18.42± 0.09Aa
A. niger 00119	5.25± 0.02Fa	12.47± 0.02Db
A. niger 00124	2.24± 0.03Ja	6.65± 0.02Jb

Means followed by the same capital letter in the column and small letter in the row do not differ from one another by the Tukey test at 5% of probability.

Table 3. Influence of pH in citric acid production by isolates of *Aspergillus niger* in submerged cultivation.

Strain	pH (BD medium)	pH (SA medium)
A. niger 00098	1.99± 0.01Da	2.47± 0.04Gb
A. niger 00100	2.30± 0.01Ga	2.96± 0.03Ib
A. niger 00102	2.44± 0.01Ha	3.02± 0.06Jb
A. niger 00104	1.81± 0.01Ba	2.16± 0.02Db
A. niger 00106	2.04± 0.02Ea	2.61± 0.04Hb
A. niger 00107	1.90± 0.01Ca	2.07± 0.02Cb
A. niger 00108	2.14± 0.02Fa	2.41± 0.01Fb
A. niger 00114	2.03± 0.01Ea	2.33± 0.02Eb
A. niger 00116	1.92± 0.02Ca	1.91± 0.02Ba
A. niger 00118	1.74± 0.04Aa	1.85± 0.02Ab
A. niger 00119	2.01± 0.02Da	2.45± 0.05Gb
A. niger 00124	2.32± 0.01Ga	2.97± 0.02Ib

Means followed by the same capital letter in the column and small letter in the row do not differ from one another by the Tukey test at 5% of probability.

The findings of Max et al. (2010) suggest that the variations in the pH values do not present significant effect for citric acid production with strains of *A. niger*. In the same way, Levinson et al. (2007), in a study with *Yarrovia lipolytica*, also found that pH does not promote significant alterations in the accumulated amount of citric acid.

In spite of all the 12 isolates solubilizing phosphate, one can realize that a somewhat higher citric acid production in the isolates (*A. niger* 00118, *A. niger* 00104, *A. niger* 00114, *A. niger* 00098, *A. niger* 00116) who presented a higher amount of soluble phosphorus and the isolates (*A niger* 00124, *A. niger* 00102, *A. niger* 00100) which had less citric acid showed less efficiency in solubilizing phosphate. Those data demonstrate the relationship between the efficiency of phosphorus solubilization and citric acid production. Therefore, greater understanding of the diversity, efficiency and capacity of microorganisms of solubilizing phosphate can further both the selection and identification of isolates potentially useful to produce citric acid.

Amplification by polymerase chain reaction (PCR)

The DNA of the isolates were extracted and amplified efficiently, nevertheless, the control (line 13) was not amplified for the ITS region. The sizes of the amplified fragments was of approximately 600 pairs of bases (bp) for all the isolates and were viewed at the same position

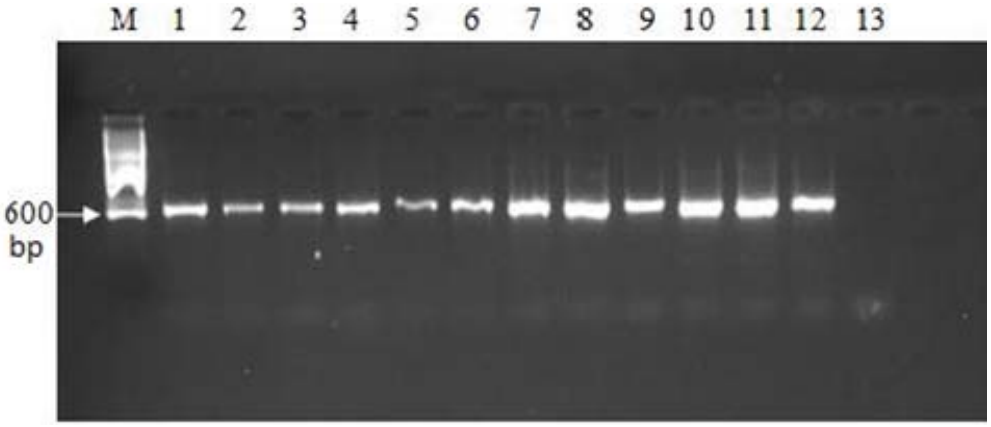

Figure 1. Product of PCR amplification of genomic DNA on agarose gel of the isolates of Asper*gillus niger*. Lines 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 represent the amplification of the genomic DNA of pure cultures of isolates of *A. niger*: 00116, 00108, 00107, 00104, 00106, 00114, 00119, 00100, 00102, 00098, 00118, and 00124, respectively and line 13 represents the control, utilizing primer ITS 1/ITS 4; line M is the molecular marker (1 Kb AMRESCO).

on the agarose gel considered of same size (Figure 1).

The 12 isolates of *A. niger* had amplified only fragments of the genomic DNA corresponding to ITS 1 and ITS 4 region with bands of about 600 pairs of base. The primers ITS 1 and ITS 4 were utilized also to warrant the quality of DNA. According to Barrocas et al. (2012) and Phan et al. (2002), the regions of ITS of rDNA have been widely utilized to distinguish and detect closely related fungal species. According to White et al. (1990), the ITS region is easily amplified, since it is comprehended between 600 and 800 pairs of base.

In the work by Menezes (2010) by means of molecular methods of DNA analysis, it was possible to distinguish species, since they detect the polymorphism existing among the sequences of nucleotides of the organisms. They exposed also in their results, the idea that the identification of species, based upon only morphological and cultural characteristics have limitations, since those are influenced by environment, altering the phenotype of fungal isolates. According to Hinrikson et al. (2005), in addition, the morphology tests are generally difficult and need staff skilled in mycology. González-Salgado et al. (2005), in a distinguishing work of *A. niger* with other species of *Aspergillus*, determined that their PCR assays based on the ITS region were both highly sensitive and unique and represent a good tool for the detection of species.

The pair of P1/P2 of citrate synthase was unique to distinguish isolates of *A. niger* in the enzyme activity. Only genomic DNA of isolates *A. niger* 00116, *A. niger* 00104, *A. niger* 00098 and *A. niger* 00118 were amplified with primers P1/P2. The size of the fragments was of about 920 pairs of bases and were viewed in the same position on the agarose gel considered of same size, the control (Line 13) being negative for amplification (Figure 2).

There are few works in the literature which define a correlation of citrate synthase of *A. niger* with citric acid production. Jaklitsch et al. (1991) in their study with enzymes involved in the citrate production by *A. niger*, evaluated citric-acid producing strains and all presented citrate synthase activity.

The tricarboxlic acid cycle plays an important role in the citric acid production by *A. niger*. On the basis of the activity of the enzymes present in fungal extract, the reaction seems to have enough natural capacity for the accumulation of citric acid (Ratledge, 2000) and an overexpression of the gene corresponding to citrate synthase, therefore, it has no effect upon the citric acid accumulation rate (Ruijter et al., 2000). So, the alterations in the citrate synthase, which occur along fermentation, should not have consequences upon citric acid production (Karaffa and Kubicek, 2003).

All the 12 isolates of *A. niger* surveyed for the analysis of genes of the citrate synthase enzyme, were selected for producing citric acid in liquid medium and only 4 of those isolates amplified their fragments for citrate synthase demonstrating a variation in the presence of the enzyme among them, that difference may be related with the fact of the isolates consisting of different sources and origins. The citrate synthase activity of isolated *A. niger* 00116, *A. niger* 00104, *A. niger* 00098, *A. niger* 00118 can be correlated with the efficient production of citric these isolates.

The citrate synthase activity of the enzyme can be considered for the natural production and accumulation of citric acid by *A. niger*, therefore, amplification of DNA is positive for the selection of different strains of microorganisms. It is necessary that further studies are developed using an induction of the production of citric acid which is used in methods to quantify the expression of citrate synthase with increased production of citric acid.

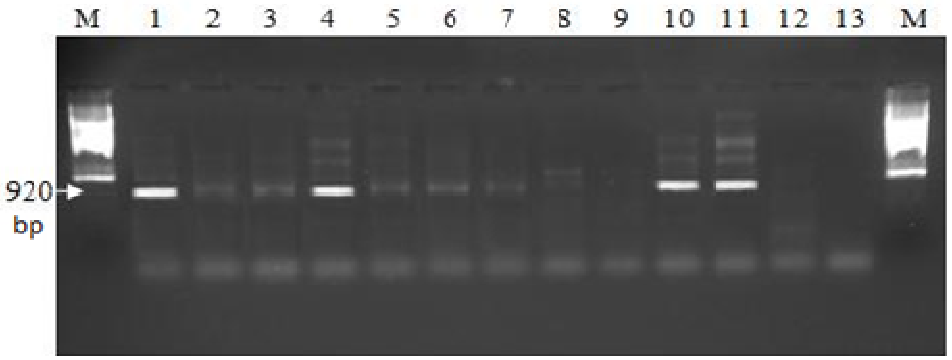

Figure 2. Product of PCR amplification of genomic DNA on agarose gel of the isolates of *Aspergillus niger*. Lines 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 represent the amplification of the genomic DNA of pure cultures of isolates of *A. niger*: 00116, 00108, 00107, 00104, 00106, 00114, 00119, 00100, 00102, 00098, 00118, and 00124, respectively and Line 13 which represents the control utilizing the pair of unique primers (P1/P2) of the citrate synthase of *Aspergillus niger*. Line M, Molecular marker (1 Kb AMRESCO).

As the real-time PCR is molecular specific, sensitive and reproducible, it may contribute to the understanding of the relationship between the enzymes of the Krebs cycle and the production of citric acid by *A. niger* and establish the biochemical mechanism and control of enzymes that inhibit or promote the process of accumulation of citric acid biosynthesis by enabling the optimization of citric acid production on an industrial scale.

Conclusions

Citric acid was one of the mechanisms of solubilizing phosphate by the isolates of *A. niger*. Isolate *A. niger* 00118 was the most efficient in the production for citric acid in the two media studied. The base sequencing of the ITS region was a simple and fast method to aid in the quality of the amplification of the DNA of the isolates of *A. niger* studied. Since the citrate synthase activity of the enzyme can be considered for the natural production and accumulation of citric acid by *A. niger*, therefore, amplification of DNA is positive for the selection of different strains of microorganisms.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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REFERENCES

Association of Official Agricultural Chemists (AOAC) (1992). Official methods of analysis of the Association of the Agricultural Chemists. 12 ed. Washington: A.O.A.C. USA.

Barrocas EN, Machado JC, Almeida MF, Botelho LS, Pinho EVRV (2012). Sensibility of the PCR technique in the detection of Stenocarpella sp. associated with maize seeds. Rev. Bras. Sement. 34:218-224.

Barroso CB, Nahas E (2005). The status of soil phosphate fractions and the ability of fungi to dissolve hardly soluble phosphates. Appl. Soil Ecol. 29:73-83.

Barroso CB, Nahas E (2008). Solubilização do fosfato de ferro em meio de cultura. Pesq. Agro. Bras. 43:529-535.

Chuang CC, Kuo YL, Chao CC, Chao WL (2007). Solubilization of inorganic phosphates and plant growth promotion by *Aspergillus niger*. Biol. Fertili. Soil 43:575-584.

Ferreira DF (2011). Sisvar: a computer statistical analysis system. Cienc. Agrotecnol. 35:1039-1042.

Gandra EA, Gandra TKV, Mello WS, Godoi HS (2008). Técnicas moleculares aplicadas à microbiologia de alimentos. Acta Scient. Technol. 30:109-118.

González-Salgado A, Patiño B, Vázquez C, Gonzáles-Jaén MT (2005). Discrimination of *Aspergillus niger* and other *Aspergillus* species belonging to section *Nigri* by PCR assays. FEMS Microbiol. Letter 245:353-361.

Hinrikson HP, Steven FH, Timothy JL, David WW, Christine JM (2005). Assessment of ribosomal large-subunit D1–D2, internal transcribed spacer 1, and internal transcribed spacer 2 regions as targets for molecular identification of medically important *Aspergillus* species. J. Clin. Microbiol. 43:2092-2103.

Jaklitsch WIM, Kubfcek CP, Scrutton MC (1991). Intracellular location of enzymes involved in citrate production by *Aspergillus niger*. Can. J. Microbiol. 37:823-827.

Karaffa L, Kubicek CP (2003). *Aspergillus niger* citric acid accumulation: do we understand this well working black box. Appl. Microbiol. Biotechnol. 61:189-196.

Kirimura K, Yoda M, Ko I, Oshida Y, Miyake K, Usami S (1999). Cloning and sequencing of the chromosomal DNA and cDNA encoding the mitochondrial citrate synthase of *Aspergillus niger*. J. Biosci. Bioeng. 88:237-243.

Levinson WE, Kurtzman CP, Kuo TM (2007). Characterization of *Yarrowia lipolytica* and related species for citric acid production from glycerol. Enzym. Microbial. Technol. 41:292-295.

Max B, Salgado JM, Rodríguez N, Cortés S, Converti A, Domínguez JM (2010). Biotechnological production of citric acid. Braz. J. Microbiol. 41:862-875.

Menezes JP (2010). Variabilidade genética na região its do rDNA de isolados de trichoderma spp. (Biocontrolador) e Fusarium oxysporum f. sp. Chrysanthemi. Ciênc. Agrotecnol. 34:132-139.
Mirhendi H, Diba K, Kordbacheh P, Jalalizand N, Makimura K (2007). Identification of pathogenic *Aspergillus* species by a PCR-restriction enzyme method. J. Medical. Microbiol. 56:1568-1570.
Murphy J, Riley JP (1962). A modified single solution method for determination of phosphate in natural waters. Analytic Chemis. Acta. 27:31-36.
Papagianni M, Mattey M (2007). Advances in citric acid fermentation by *Aspergillus niger*: biochemical aspects, membrane transport and modeling. Biotechnol. Advanc. 25:244-263.
Phan HTT, Ford R, Bretag T, Taylor PWJ (2002). A rapid and sensitive polymerase chain reaction (PCR) assay for detection os *Ascochyta rabiei*, the cause of ascochyta blight of chickpea. Austral. Plant. Pathol. 31:31-39.
Pitt JI, Hocking AD (1997). *Fungi* and food spoilage. London. Chapman & Hall. p.593.
Prado FC, Vandenberghe LPS, Soccol CR (2005). Relation between citric acid production by solid-state fermentation from cassava bagasse and respiration of *Aspergillus niger* LPB 21 in semi-pilot scale. Braz. Arch. Biol. Technol. 48:29-36.
Ratledge C (2000). Look before you clone: a comment on 'properties of *Aspergillus niger* citrate synthase and effects of citA overexpression on citric acid production' by Ruijter, Panneman, Xu and Visser. FEMS Microbiol. Letter. 189:317-319.
Ruijter GJG, Panneman H, Xu DB, Visser J (2000). Properties of *Aspergillus niger* citrate synthase and effects of citA overexpression on citric acid production. FEMS Microbiol. Letter. 184:35-40.
Soccol CR, Vandenberghe LP, Rodrigues C, Pandey A (2006). New perspectives for citric acid production and application. Food Technol. Biotechnol. 44:141.
Vassilev N, Medina A, Azcon R, Vassileva M (2006). Microbial solubilization of rock phosphate on media containing agro-industrial wastes and effect of the resulting products on plant growth and P uptake. Plant Soil. 287:77-84.
White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequences of fungal ribossomal RNA genes for phylogenetics. In: INNIS, M. A. et al. (Ed.). PCR protocols: a guide to methods and applications. San Diego Academic. pp.315-322.