

## Enzymatic Activity of Endophytic Bacterial Isolates of *Jacaranda decurrens* Cham. (Carobinha-do-campo)

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### ABSTRACT

*The objectives of this work was to isolate endophytic bacteria from Jacaranda decurrens Cham. and screening of some enzymes of biotechnological interest. Ten (10) bacterial species were isolated and identified from the leaves and stems. All the isolates presented enzymatic activity, which was ranked as follows: proteolytic (60%) and amilolytic activity (60%), lipolytic (40%), esterase (40%). However, cellulolytic and pectinolytic activities were not detected. This is the first report on the isolation and identification of endophytic bacteria from Jacaranda decurrens Cham.*

**Key words:** Endophytic bacteria, *Jacaranda decurrens*, enzymes

### INTRODUCTION

Endophytic microorganisms (fungi and bacteria) are those that inhabit inside of a plant at least in a period of its vital cycle, and are found in tissues such as leaves, branches and roots. Apparently, they do not cause any damage to the host, which distinguishes them from the phytopatogens microorganisms (Azevedo et al., 1999). Their presence implied a symbiotic interaction, in all the plants investigated until now (Azevedo et al., 2000). They were mentioned for the first time by Bary in 19<sup>th</sup> century (Azevedo, 1998), gaining interest from 1970's. This interest is due to the possibility of these microorganisms producing pharmacologically active substances with biotechnological potential such as antitumor agents (*Pestalotiopsis microspora*, taxol), antifungal agents (*Cryptosporiopsis criptocandina*,

quercine), besides producing factors of plant growth, toxins and enzymes, including some microorganisms being used as biological controllers of many diseases and plagues. (Araújo et al., 2002; Strobel, 2002; Azevedo, 1998; Stierle et al., 1993).

The importance of the microorganisms in enzyme production is due its high production capability, low cost and susceptibility to genetic manipulation. Actually, the enzymes of microbial origin have high biotechnological interest such as in the processing of foods, manufacturing of detergents, textiles, pharmaceutical products, medical therapy and in molecular biology (Pilnik and Rombouts, 1985; Falch, 1991; Rao et al., 1998). It is, therefore, necessary to find microorganisms that produce enzymes for specific substrate, with different requirements in temperature range, pH and presence of different

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ions, for different production processes (Falch, 1991). The endophytic microorganisms occupy a relatively unexplored site in microorganism isolation so they can represent a new source in obtaining more enzymes with different potentialities.

The species *Jacaranda decurrens* Cham. is a shrub present in diverse physiognomies of the Brazilian Cerrado (Prado et al., 2002), popularly described as a medicinal plant that act in gynaecological infections, giardiasis and amoeba's diseases and as an anti-syphilitic. The aim of this work was the isolation of endophytic microorganisms from *Jacaranda decurrens* Cham and the evaluation of enzyme production such as amylase, esterase, lipase, protease, pectinase and cellulose, by the isolates.

## MATERIALS AND METHODS

### Isolation of the microorganisms

Leaves and stems parts of one adult *Jacaranda decurrens* (about 2 years old) with healthy appearances were collected once from the vegetables garden of the Federal University of Goiás (UFG). The samples were disinfected according to Araújo et al. (2002), washed immediately with water for removal of dust and soil, in absorbent paper and cut in fragments with about 0.5 cm wide. The fragments were successively treated with ethanol 70% (1 min), sodium hypochlorite 2-4% (available chlorine) for 4 min. and exhaustively rinsed with sterilized distilled water. The fragments were then submitted in ultra-sound bathing (40 KHz) for 10 min., then rinsed again with sterilized distilled water for 3 times and submitted again to a bathing with ethanol 70% for 30 seconds and washed twice with sterilized distilled water. Aliquots of 1.0 mL of the last wash water were inoculated in 9.0 mL of nutrient broth in order to evaluate the effectiveness of the disinfection process.

After the disinfection, the samples were transferred to Petri dishes containing water-agar medium (1.8% agar), nutrient agar with peptone, tryptic soy agar, King's media, starch-casein agar and ISP-2 agar with 1% of starch; all added with Nistatin (200 µg mL<sup>-1</sup>) and replicated four times. The plates were incubated at 30°C for 30 days (Azevedo et al., 2000). The colonies obtained were successively cut-up in each medium. This process

was repeated five times and was accompanied by the description of the morphology and stained by Gram method. The isolates obtained were then grown in flasks containing nutrient agar and stored in refrigerator at 4°C. At the same time, the isolates were maintained at -20°C in cryogenic tubes containing glycerol at 50% in freezer.

### Identification of the microorganisms

The morphological and stain identification was made through the Gram stain method and through the RYU reaction (Ryu, 1938, 1940). The isolates were then identified through commercial biochemical kits. For the identification of *Enterobacteriaceae* and Gram-negative non fermenters BBL Crystal TM Enteric/Nonfermenter (E/NF) Identification (ID) System with 30 reactions were used and for Gram-positive bacteria, BBL Crystal TM Gram-positive (GP) Identification (ID) System with 29 reactions were used.

### Determination of the enzymatic activity

The productions of following enzymes were analysed: amylase, esterase, lipase, cellulase, protease and pectinase. The enzymatic activities were performed by initially growing the isolates in BHI (brain heart infusion) broth for 24 h. at 30°C. After that, aliquots of 100 µl were inoculated on the specific culture media for each enzyme to be investigated. The cultures were incubated at 30°C during 96 h.. The Enzymatic Index (EI) was determined within 24, 48, 72 and 96 h. incubation, according to specific methodologies for each investigated enzyme. The Enzymatic Index (EI) was expressed by the relationship between the average diameter of the degradation halo and the average diameter of the colony growth (Hankin et al., 1971; Hankin and Anagnostakis, 1975).

### Determination of amilolytic activity

The methodology used was described by Hankin and Anagnostakis (1975). The isolates were inoculated in nutrient agar (NA) with 0.2% of soluble starch (g l<sup>-1</sup>), pH 6.0. After incubation, the cultures were treated under iodine vapours, which allowed the visualization of clear halos around the colonies.

### Determination of the esterase activity

The media used was as described by Sierra (1957), containing (g l<sup>-1</sup>): peptone 10.0, NaCl 5.0, CaCl<sub>2</sub>

2H<sub>2</sub>O 0.1, agar 18.0, pH 7.4. To the sterilized culture media, previously sterilized Tween 80 was added in a final concentration of 1% (v/v). This medium was inoculated with the isolates and the presence of halos observed.

#### Determination of the lipolytic activity

A previously described methodology was used to determine the esterase activity (Sierra 1957). In this determination, Tween 80 was substituted by Tween 20. The presence of clear halos around the colonies was observed.

#### Determination of the proteolytic activity

To determine the casein hydrolysis we used (g l<sup>-1</sup>) nutrient broth 8.0, glucose 1.0, and agar 18.0, pH 7.4 (Vieira, 1999). After autoclaving, 15.0 ml of skimmed milk, separately autoclaved was added. The microorganisms were inoculated and after the growth period 2.0 ml of HCl 0.1 mol l<sup>-1</sup> was added to the plates and the presence of clear halos around the colonies were observed. In another method, the hydrolysis of gelatine was determined using the media of Frazier's gelatin agar, containing nutrient agar (pH 7.0) and bacteriological gelatin (4.0 g l<sup>-1</sup>). After incubation at 30°C for 72 h., the plates were covered with Frazier's revealers (distilled water 100 ml, HCl 20.0 ml and mercury dichloride 15.0 g) (Smibert and Krieg, 1994). The presence of a clear halo around the bacterial growth was observed.

#### Determination of the cellulolytic activity

Two methodologies were used for. In the first method, the cellulolytic activity was determined incubating the plates at 50°C, over night, to facilitate the visualization of halo degradation of the cellulose (Stamford et al., 1998). The second method was according to Samanta et al. (1989).

The plates were incubated for eight days at 30°C and the presence of a clear halo around the bacterial growth was observed.

#### Determination of the pectinolytic activity

Two methodologies for the determination of this enzymatic activity were used. The first one was as described by Hankin et al. (1971). After the incubation period (96 h.) the plates were recovered by a CTAB solution (hexadecyl trimethyl ammonium bromide) 1% (p/v) and incubated at temperature for room. After this, the plates were washed with sterilized distilled water and the degradation of the pectin (clear halos around the colonies) was determined. Second method was of Andro et al. (1984). The plates were inoculated and incubated at 30°C for 96 h. After this, 5.0 ml of HCl (2 mol l<sup>-1</sup>) was added to the plates and the presence of clear halo around the colonies was indicative of the degradation of pectin. The experiments were performed using five repetitions and the results were analyzed statistically through the variance test and the averages compared by the test *t*, the tolerance level being set at 95%, making of the program Statistica' 99, version 5.5, produced by StatSoft Inc.

## RESULTS

### Endophytic isolates

A total of 18 microorganisms were isolated from leaves and stem of *Jacaranda decurrens*. After the identification, the isolates were grouped in 10 species (Table 1) with predomination of the bacteria of the genera *Bacillus* (39%), *Pseudomonas* (27.6%), *Corynebacterium* (16.7%), *Actinomyces* (11.1%) and *Staphylococcus* (5.6%).

**Table 1** - Endophytic microorganisms isolated from *Jacaranda decurrens*

Isolated microorganisms	Number of Isolates (%)
<i>Actinomyces pyogenes</i>	2 (11.11)
<i>Bacillus circulans</i>	1 (5.55)
<i>Bacillus coagulans</i>	1 (5.55)
<i>Bacillus licheniformis</i>	2 (11.11)
<i>Bacillus megaterium</i>	2 (11.11)
<i>Corynebacterium aquaticum</i>	2 (11.11)
<i>Corynebacterium renale</i>	1 (5.55)
<i>Pseudomonas stutzeri</i>	5 (27.77)
<i>Staphylococcus</i> sp	1 (5.55)
<i>Bacillus</i> sp	1 (5.55)
<b>Total</b>	<b>18 (100)</b>

### Enzymatic activity

In Table 2, the results of Enzymatic Index (EI) of the isolates tested are shown. Amylolytic activity was observed in the isolates CAR1, CAR3, CAR4, CAR5 CAR8 and CAR9, representing 60% of the samples. The isolate CAR8 (EI 2.6) presented a significant difference at the level of  $p < 0.05$  for the test of  $t$  of Student when compared to others. Esterasic and lipase activities were presents in 40% of the isolates (Table 2), isolate CAR7 presented EI of 6.2 and 3.7, respectively, with significant difference at the level of  $p < 0.05$  for

the test of  $t$  of Student when compared with the other isolates (Table 2).

It was observed that 30 and 60% of the isolates were able to hydrolyse casein and gelatin, respectively. Isolate CAR7 presented EI of 4.3 and 4.6 for casein and gelatin hydrolysis, respectively. These results presented significant difference at level of  $p < 0.05$  for the test of  $t$  of Student when compared with the other isolates (Table 2). Activity for pectinase and cellulase was not observed among all of the species studied.

**Table 2** - Enzymatic activity of the isolated microorganisms of *Jacaranda decurrens*<sup>†</sup>.

Isolated microorganisms		Enzymatic Index (EI) <sup>*</sup>				
		Amylase	Esterase (T80)	Lipase (T20)	Protease (Casein)	Protease (Gelatin)
<i>Actinomyces pyogenes</i>	CAR1	1.2 <sup>d</sup>	0	0	2.0 <sup>c</sup>	2.6 <sup>b</sup>
<i>Bacillus circulans</i>	CAR2	0	0	0	0	4.4 <sup>a</sup>
<i>Bacillus coagulans</i>	CAR3	1.7 <sup>c</sup>	0	0	0	2.0 <sup>c</sup>
<i>Bacillus licheniformis</i>	CAR4	1.8 <sup>b,c</sup>	0	0	0	1.9 <sup>c</sup>
<i>Bacillus megaterium</i>	CAR5	2.2 <sup>a,b</sup>	0	0	3.1 <sup>b</sup>	2.8 <sup>b</sup>
<i>Corynebacterium aquaticum</i>	CAR6	0	3.0 <sup>b</sup>	2.9 <sup>b</sup>	0	0
<i>Corynebacterium renale</i>	CAR7	0	6.2 <sup>a</sup>	3.7 <sup>a</sup>	4.3 <sup>a</sup>	4.6 <sup>a</sup>
<i>Pseudomonas stutzeri</i>	CAR8	2.6 <sup>a</sup>	3.2 <sup>b</sup>	2.4 <sup>c</sup>	0	0
<i>Staphylococcus</i> sp	CAR9	1.8 <sup>b,c</sup>	2.8 <sup>b</sup>	2.9 <sup>b</sup>	0	0
<i>Bacillus</i> sp	CAR10	0	0	0	0	0

\* The enzymatic index represents the halo diameter of degradation/diameter of colony in cm. Averages followed by the same letter don't differ among themselves (test  $t$  of Student,  $p < 0.05$ ).

† An average of five repetitions.

## DISCUSSION

Bacteria are common inhabitants on the surface as well as inside tissues of the plants, exerting various effects on the development of these (Selosse et al., 2004). The bacteria that live in the interior of plants without causing diseases to their hosts are called endophytic (Petrini et al., 1989; Azevedo et al., 2000). Endophytic microorganisms have been isolated from different types of vegetation such as grass (Naffaa et al., 1998), corn (Araújo et al., 2000; McInroy and Kloepper, 1995), *Eriaceae* (Petrini, 1984), cotton (McInroy and Kloepper, 1995), bryophytes (Costa et al., 2001), *Solanum lycocarpum* (Maitan, 1998) and agricultural crops (Halmann et al., 1997). Studies for the isolation and characterization of endophytic microorganisms of native plant specimens from Brazilian Cerrado biome are rare. This is therefore, the first report of the isolation, identification and characterization of endophytic microorganisms of

the specimen of the *Bignoniaceae* family, *Jacaranda decurrens* Cham.

The results obtained shown the common phenomenon of association between the microorganisms and plants (Azevedo, 1998). A variety of genera and species of bacteria, were observed. Apparently, this is second report for the isolation of *Staphylococcus* sp as endophytic bacteria (Stamford et al., 1998).

The research with endophytic microorganisms is more focused in species with economic value such as corn, soy, grasses and others (Petrini, 1984; McInroy and Kloepper, 1995; Halmann et al., 1997; Naffaa et al., 1998; Araújo et al., 2000; Costa et al., 2001) or species capable of producing antimicrobials, as *Taxus wallachiana* (Yang et al., 1994).

The method of the radial diffusion in solid media indicated the activity in qualitative form, directly correlating the diameters of the halo of degradation and the colony (Enzymatic Index) (Lin et al., 1991). The Enzymatic Index is a

practical tool that facilitates and speeds the selection and the comparison of the enzymatic production of different isolates microbial, once in this index direct consideration the correlation are taken between the size of the halo and the degradation capacity of the microorganisms (Lin et al., 1991; Fungaro and Maccheroni 2002). Fungaro and Maccheroni (2002) suggested that Enzymatic Index larger than 1.0 were indicative of excretion of enzymes.

It was observed that all the isolates possessed at least one enzymatic activity tested (Table 2). The amylolytic activity was observed for isolates *B. coagulans* CAR3, *B. licheniformis* CAR4, *B. megaterium* CAR5, *Act. pyogenes* CAR1 and *P. stutzeri* CAR8. So far only *Ps. stutzeri*, *B. megaterium* and *B. licheniformis* have been suggested as producers of extracellular amylase (Schmidt and John, 1979; Rivera et al., 2003). These results suggested that isolates *Act. pyogenes* CAR1 and *B. coagulans* CAR3, produced extracellular amylolytic enzymes.

Among the producers of esterases and lipases, *Bacillus* as well as *Ps. stutzeri* have been described (Jung et al., 2003; Ruiz et al., 2002). Species of *Corynebacterium* have not been described as potential producers of esterases and lipases (Hou and Johnston, 1992), however, in the results obtained it was observed that the isolate *Coryne. renale* CAR 7 demonstrated the best EI for esterase and lipase (6.2 and 3.7, respectively), suggesting the potential of this isolate as producer of these enzymes (Jaeger and Eggert, 2002). The best proteolytic production was observed in gelatinase producing bacteria compared to the casein producers, although CAR1, CAR5 and CAR7 produced both enzymes. Apparently bacteria produced unspecific proteases capable to hydrolyze both casein and gelatin. CAR2, CAR3 and CAR4 hydrolyzed gelatin only, suggesting that this enzyme was more specific for collagen.

The presence of proteolytic activity in *B. circulans* CAR 2, *B. coagulans* CAR3, *B. licheniformis* CAR4, *B. megaterium* CAR5 and *Coryne. renale* CAR7 confirmed that some species of *Bacillus* and *Corynebacterium* tended to synthesize proteolytic enzymes during the sporulation process (Ponnuraj et al., 1999; Kamasaka et al., 2002), as observed in this study. Ryttersgaard et al. (2004) mentioned pectinase production by *B. liqueniformis* and Sorian et al. (2005) for *Bacillus* sp, but in this work these species did not show the same enzyme

production, despite using two different growth media.

This is the first report on the isolation of endophytic microorganisms with production of enzymatic activity in solid media. *Jacaranda decurrens* Cham as the native plant of Brazilian Cerrado and used in popular phytoterapy, it revealed new endophytic source with applicable biotechnological potential in different areas such as in the nutrition, detergent, paper, pharmaceutical, textile and leather industries.

## RESUMO

O objetivo deste trabalho foi isolar bactérias endofíticas de *Jacaranda decurrens* Cham. e selecionar algumas enzimas de interesse biotecnológico. Dez espécies de bactérias foram isoladas e identificadas de caule e folhas. Todos os isolados de apresentaram atividade enzimática, e a maior predominância foi de atividade proteolítica (60%) e atividade amilolítica (60%), seguidas atividades lipolítica (40%) e esterásica (40%). Contudo, as atividades celulolítica e pectinolítica não foram detectadas. Este é o primeiro relato de isolamento e identificação de bactérias presentes em *Jacaranda decurrens* Cham.

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