Biotechnology and Industrial Microbiology

The isolation of pentose-assimilating yeasts and their xylose fermentation potential

Gisele Marta Martins, Daniela Alonso Bocchini-Martins, Carolina Bezzerra-Bussoli, Fernando Carlos Pagnocca, Maurício Boscolo, Diego Alves Monteiro, Roberto da Silva, Eleni Gomes

a Universidade Estadual Paulista (UNESP), Instituto de Pesquisa em Bioenergia-IPBen, Laboratório de Microbiologia aplicada, São José do Rio Preto, SP, Brazil
b Universidade Estadual Paulista (UNESP), Instituto de Química, Araraquara, SP, Brazil
c Universidade Estadual Paulista-UNESP, Centro de Estudos de Insetos Sociais-CEis, Campus de Rio Claro, SP, Brazil

A R T I C L E   I N F O

Article history: Received 5 May 2016 Accepted 13 November 2016 Available online 26 August 2017 Associate Editor: Solange I. Mussatto

Keywords: Xylose Yeasts Ethanol

A B S T R A C T

For the implementation of cellulosic ethanol technology, the maximum use of lignocellulosic materials is important to increase efficiency and to reduce costs. In this context, appropriate use of the pentose released by hemicellulose hydrolysis could improve economic viability of this process. Since the Saccharomyces cerevisiae is unable to ferment the pentose, the search for pentose-fermenting microorganisms could be an alternative. In this work, the isolation of yeast strains from decaying vegetal materials, flowers, fruits and insects and their application for assimilation and alcoholic fermentation of xylose were carried out. From a total of 30 isolated strains, 12 were able to assimilate 30 g L⁻¹ of xylose in 120 h. The strain Candida tropicalis S4 produced 6 g L⁻¹ of ethanol from 56 g L⁻¹ of xylose, while the strain C. tropicalis E2 produced 22 g L⁻¹ of xyitol. The strains Candida oleophila G10.1 and Metschnikowia korensis G18 consumed significant amount of xylose in aerobic cultivation releasing non-identified metabolites. The different materials in environment were source for pentose-assimilating yeast with variable metabolic profile.

© 2017 Published by Elsevier Editora Ltda. on behalf of Sociedade Brasileira de Microbiologia. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Introduction

Bioethanol or first generation ethanol in Brazil is obtained by fermenting glucose from sugar cane juice and molasses (a residue from sugar making) using Saccharomyces cerevisiae. While this technology is a consolidated industrial process, production of second generation ethanol remains a challenge. One of the bottlenecks is the utilization of pentoses released by hydrolysis of hemicellulose that correspond to around 30% of the lignocellulosic feedstock. Yeasts able to convert xylose into ethanol have been described like as Scheffersomyces (Pichia) stipitis, S. (Candida) shehatae and Pachysolen tamophilus. However, the yield has not reached a satisfactory level for industrial...
applications. On the other hand, pentose-fermenting yeasts can be used for obtaining of high aggregate value products. The pentose-fermenting capacity of yeasts Pseudoyzyma antarctica FYCC 5048T, P. aphidis FYCC 5535T and P. rugulosa FYCC 5537T and production of a glycolipid biosurfactant was demonstrated as well as the production of xylitol and arabinol by Debaryomyces Hansenii from pentose. A number of recent studies have been focused on the genetic engineering of S. cerevisiae, aimed at making it able to produce ethanol from glucose and xylose. Nevertheless, the yield achieved resembles the xylene-fermenting species, the xylose utilization is slow and occurs only after glucose exhaustion. The search for genetically modified strains is the focus of several studies. Thus, the study of microorganisms pentose-fermenting and isolation of genes involved in pentose fermentation can afford support for these studies.

Another research tendency is the use of pentose to produce alternative compounds like organic acids, lipids for biodiesel production, isobutanol and hydrogen all using mainly yeast and bacteria. Besides compounds already cited, new alternative products with high aggregate value, are still appearing, increasing the options for the utilization of these sugars in biorefineries system.

Considering the importance of the use of pentoses from lignocellulosic materials, the present work aimed at isolating yeast strains with the ability to assimilate and ferment xylose for future use for obtaining of products with biotechnological interest. Thus, this study contributes by increasing the information on xylose-fermenting strains and offers genomes for future studies of the metabolism of microorganisms.

Materials and methods

Isolation and identification of strains

The strains were isolated from fruits, flowers, insects and grape residues. About 0.5 g of material was suspended in 4 mL of YEPX medium (1% of yeast extract, 2% of peptone and of 2% xylose) and incubated at 30 °C. A loop of the homogenized culture was then streaked on the solid YEPX medium in Petri dishes to isolate the colonies. The strain identification was made using sequences of the D1/D2 domains of the rDNA.

Xylose assimilation assay

To evaluate the potential of xylose assimilation, the strains were pre-cultivated in a YEP medium (1% yeast extract, 2% peptone and 2% glucose) and the biomass was centrifuged, washed twice with sterile distilled water and a suspension of them used as inoculum at 1 g of dry biomass per liter of culture medium composed of KH2PO4 (2.0 g L−1), (NH4)2SO4 (2.0 g L−1), MgSO4 · 7H2O (1.0 g L−1), urea (0.3 g L−1), CaCl2 (0.3 g L−1) and xylose (30.0 g L−1). The incubation was carried out at 30 °C and 150 rpm. The assays were done in duplicate and with three repetitions.

Ethanol production assay

The alcoholic fermentation was assayed in 125 mL Erlenmeyer flasks adapted for alcoholic fermentation, closed with a valve containing sodium metabisulfite solution at 1 g L−1 to ensure that no oxygen got, and containing 60 mL of medium described above but with yeast extract at 10 g L−1 and xylose at 100.0 g L−1. The incubation was at 30 °C. The assays were done in duplicate with three repetitions.

Analytical methods

To evaluate the dry cell mass, samples of fermented medium were centrifuged at 10,000 × g, by 15 min, the supernatant was discarded and the precipitated cells were dried at 60 °C until they reached constant weight.

The reducing sugar concentration was determined using the dinitrosalicylic (DNS) acid method, based on Miller (1959).

The ethanol concentration was measured by a gas chromatograph (HP 5890) with an FFAP capillary column (polypeylene glycol – 30 mm × 0.22 mm × 0.3 μm) and a flame ionization detector, one split/splitless injector. Nitrogen was utilized as a carrier gas at 30 mL min−1. Temperatures at injector and detector were 250 °C.

The xylitol quantification was performed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). All samples were filtered (0.22 μm membrane) and injected (20 μL) in HPAEC-PAD System (ICS, Dionex Corporation, USA) equipped with automatic sampler AS40. The form of wave pattern used was the standard quadruple with the following potential pulse and durations: E1 = 0.10 V (t1 = 0.40 s); E2 = −2.00 V (t2 = 0.02 s); E3 = 0.60 V (t3 = 0.01 s); E4 = −0.10 V (t4 = 0.06 s). An Isocratic run was performed with 10 mM NaOH at a flow rate of 1 mL min−1 and 35 °C.

Results

Isolation, identification of yeasts and evaluation of xylose assimilation

A total of 30 strains were isolated and able to grow on the liquid YEPX medium, but, among those, four were not able to grow in liquid medium with xylose as the sole carbon source (Table 1). In aerobic cultivation, nine strains consumed all the xylose present in the medium (30 g L−1) in 120 h.

The strain Pichia guilliermondii G1.2 was the most efficient in the xylose consumption (maximum at 48 h) while strains Candida tropicalis FP, C. tropicalis S4 and P. guilliermondii G4.2 exhausted the sugar in 72 h.

Species or genera isolated in this work, such as Asperosidium pullulans, Issatchenka terricella, I. orientalis, Metschnikowia bicuspidata, M. chrysosporiae, M. zobellii, P. guilliermondii, P. stipititis, Rhodotorula minuta and R. mucilaginosa are mentioned in the literature as being able to produce ethanol from xylose. The genera Hanseniaspora and the specie I. terricella are glucose fermenting, but there is contradicting information about
Table 1 – Yeasts isolated and xylose consumption during aerobic cultivation using a basal medium and xylose (30 g L⁻¹) as the sole carbon source.

<table>
<thead>
<tr>
<th>Isolated strains</th>
<th>Samples</th>
<th>Xylose consumption (g L⁻¹)</th>
<th>Time (h)</th>
<th>Maximum biomass production (g L⁻¹)</th>
<th>Time (h)</th>
<th>Yield (biomass/xylose) (g g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aureobasidium pullulans</em> G19</td>
<td>Flor (Nyctaginaceae)</td>
<td>9.9</td>
<td>120</td>
<td>9.7</td>
<td>120</td>
<td>0.9</td>
</tr>
<tr>
<td><em>Candida tropicalis</em> E2</td>
<td>Grape residue</td>
<td>30.0</td>
<td>120</td>
<td>8.0</td>
<td>120</td>
<td>0.2</td>
</tr>
<tr>
<td><em>C. tropicalis</em> FP</td>
<td>Anthill</td>
<td>30.0</td>
<td>72</td>
<td>7.2</td>
<td>120</td>
<td>0.2</td>
</tr>
<tr>
<td><em>C. tropicalis</em> S2</td>
<td>Grape leaf</td>
<td>10.0</td>
<td>120</td>
<td>3.3</td>
<td>96</td>
<td>0.3</td>
</tr>
<tr>
<td><em>C. tropicalis</em> S3</td>
<td>Grape</td>
<td>10.0</td>
<td>120</td>
<td>3.1</td>
<td>120</td>
<td>0.3</td>
</tr>
<tr>
<td><em>C. tropicalis</em> S4</td>
<td>Grape leaf</td>
<td>30.0</td>
<td>72</td>
<td>7.3</td>
<td>120</td>
<td>0.2</td>
</tr>
<tr>
<td><em>C. tropicalis</em> T</td>
<td>Wine must</td>
<td>20.1</td>
<td>120</td>
<td>7.1</td>
<td>120</td>
<td>0.3</td>
</tr>
<tr>
<td><em>C. oleophila</em> G10.1</td>
<td>Leaf (Asteraceae)</td>
<td>30.0</td>
<td>96</td>
<td>8.2</td>
<td>120</td>
<td>0.3</td>
</tr>
<tr>
<td><em>Hanseniaspora guilliermondii</em> G9.1</td>
<td>Leaf (Asteraceae)</td>
<td>7.6</td>
<td>120</td>
<td>2.6</td>
<td>96</td>
<td>0.3</td>
</tr>
<tr>
<td><em>Hanseniaspora</em> sp. G1.3</td>
<td>Fruit (Anarcardiaceae)</td>
<td>19.4</td>
<td>120</td>
<td>3.7</td>
<td>72</td>
<td>0.2</td>
</tr>
<tr>
<td><em>Hanseniaspora</em> sp. G11.1</td>
<td>Fruit (Rutaceae)</td>
<td>7.6</td>
<td>120</td>
<td>3.5</td>
<td>120</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Hanseniaspora</em> sp. G13</td>
<td>Fruit (Rutaceae)</td>
<td>10.4</td>
<td>120</td>
<td>2.7</td>
<td>72</td>
<td>0.2</td>
</tr>
<tr>
<td><em>Hanseniaspora</em> sp. G14</td>
<td>Flower (Myrtaceae)</td>
<td>9.9</td>
<td>120</td>
<td>2.0</td>
<td>120</td>
<td>0.2</td>
</tr>
<tr>
<td><em>Hanseniaspora</em> sp. G15</td>
<td>Fruit (Malpighiaceae)</td>
<td>3.5</td>
<td>120</td>
<td>2.2</td>
<td>96</td>
<td>0.6</td>
</tr>
<tr>
<td><em>Hanseniaspora</em> sp. G17</td>
<td>Flower (Rubiaceae)</td>
<td>5.4</td>
<td>120</td>
<td>2.8</td>
<td>48</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Hanseniaspora</em> sp. G2</td>
<td>Flower (Amaryllidaceae)</td>
<td>10.2</td>
<td>120</td>
<td>5.6</td>
<td>48</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Hanseniaspora</em> sp. G4.1</td>
<td>Flower (Nyctaginaceae)</td>
<td>23.0</td>
<td>120</td>
<td>8.2</td>
<td>120</td>
<td>0.3</td>
</tr>
<tr>
<td><em>Hanseniaspora</em> sp. G7.1</td>
<td>Fruit (Myrtaceae)</td>
<td>23.9</td>
<td>120</td>
<td>8.1</td>
<td>120</td>
<td>0.3</td>
</tr>
<tr>
<td><em>Issatchenkia terricola</em> G20</td>
<td>Fruit (Myrtaceae)</td>
<td>5.1</td>
<td>120</td>
<td>5.0</td>
<td>96</td>
<td>0.9</td>
</tr>
<tr>
<td><em>Metschnikowia koreensis</em> G18</td>
<td>Flower (Amaryllidaceae)</td>
<td>30.0</td>
<td>120</td>
<td>6.7</td>
<td>96</td>
<td>0.2</td>
</tr>
<tr>
<td><em>Pichia guilliermondii</em> G4.2</td>
<td>Fruit (Anarcardiaceae)</td>
<td>30.0</td>
<td>48</td>
<td>10.8</td>
<td>120</td>
<td>0.3</td>
</tr>
<tr>
<td><em>Pichia guillarmondii</em> G4.2</td>
<td>Fruit (Rosaceae)</td>
<td>30.0</td>
<td>72</td>
<td>10.6</td>
<td>120</td>
<td>0.3</td>
</tr>
<tr>
<td>P. guillermondii B1</td>
<td>Wine must</td>
<td>nd</td>
<td>–</td>
<td>nd</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>P. guillermondii B2</td>
<td>Grape</td>
<td>nd</td>
<td>–</td>
<td>nd</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>P. kluveri G1.1</td>
<td>Fruit (Anarcardiaceae)</td>
<td>20.5</td>
<td>120</td>
<td>7.2</td>
<td>120</td>
<td>0.3</td>
</tr>
<tr>
<td><em>Rhodotorula mucilaginosa</em> G7.2</td>
<td>Flower (Euphorbiaceae)</td>
<td>29.4</td>
<td>120</td>
<td>11.5</td>
<td>120</td>
<td>0.4</td>
</tr>
<tr>
<td>R. mucilaginosa G11.2</td>
<td>Flower (Plantaginaceae)</td>
<td>17.3</td>
<td>120</td>
<td>6.7</td>
<td>120</td>
<td>0.4</td>
</tr>
<tr>
<td><em>Rhodotorula sp. G10.2</em></td>
<td>Flower (Plantaginaceae)</td>
<td>27.8</td>
<td>120</td>
<td>8.1</td>
<td>120</td>
<td>0.3</td>
</tr>
<tr>
<td>Yarrowia lipolitica CO1</td>
<td>Grape</td>
<td>nd</td>
<td>–</td>
<td>nd</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Yarrowia lipolitica CO2</td>
<td>Grape</td>
<td>nd</td>
<td>–</td>
<td>nd</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

- Identification based on sequences of the D1/D2 domains of the rDNA (at least 98% similarity, with reference to homologous sequences from GenBank).
- Not quantified, the strains were not able to grow with xylose as the sole carbon source.

The xylose assimilation by these yeasts. The most efficient xylose-assimilating yeasts were chosen to continue the experiments and used for fermentation for ethanol production in subsequent assays.

**Ethanol production by yeasts**

The ethanol production was evaluated for 30 strains and four of them were able to produce ethanol from xylose (from 3 to 6 g L⁻¹) (data not shown). The three strains of *C. tropicalis* showed different profiles of consumption of xylose and ethanol production. The highest ethanol production was observed with *C. tropicalis* S4 with 6 g L⁻¹ of ethanol and whose xylose assimilation was 56 g L⁻¹ resulting in a yield of 0.1 g g⁻¹, following by *C. tropicalis* FP that produced 4.6 g L⁻¹ of ethanol from 84 g L⁻¹ of xylose (yield of 0.05 g g⁻¹).

The strains *Rhodotorula* sp. G10.2 and *C. tropicalis* E2 showed similar production of ethanol (around 3 g L⁻¹) to each other but *Rhodotorula* sp. G10.2 consumed 73 g L⁻¹ of xylose with yield of 0.04 g g⁻¹ and *C. tropicalis* E2 consumed 53 g L⁻¹ with yield of 0.05 g g⁻¹ (Fig. 1A).

**Fig. 1B** shows the xylose assimilation and biomass production bynotin able to produce ethanol. *P. guillermondii* G4.2, *C. oleophila* G10.1 and *M. koreensis* G18 showed yields of biomass of 0.7 g g⁻¹ meanwhile *P. guillermondii* G1.2 and *R. mucilaginosa* G7.2 had yields of 1.1 up to 1.3 g g⁻¹, suggesting that *P. guillermondii* G4.2, *C. oleophila* G10.1 and *M. koreensis* G18 produced not identified metabolite.

Since *C. tropicalis* has been reported as a xylitol-producing species and taking into account its consumption of xylose, the xylitol quantification in the fermentation media was carried out (Table 2).

The strain *C. tropicalis* E2 reached a production of 22 g L⁻¹ of xylitol from 100 g L⁻¹ of xylose (yield of 0.2 g g⁻¹) while *S4 produced 15.5 g L⁻¹ of xylitol (0.15 g g⁻¹) and *C. tropicalis* FP produced 4.5 g L⁻¹ of xylitol (0.04 g g⁻¹). The comparison between xylitol and ethanol fermentative parameters shows that the strain *C. tropicalis* E2 exhibits more potential for xylitol production while *C. tropicalis* FP produced similar concentrations of both alcohols but with low yield. In the culture medium of strain *C. tropicalis* S4 a higher concentration of xylitol than ethanol was measured.
Fig. 1 – Alcoholic fermentation assay in a basal medium with xylose (100 g L\(^{-1}\)) and yeast extract (10 g L\(^{-1}\)). (A) Ethanol production and xylose consumption by C. tropicalis E2 (squares), C. tropicalis S4 (circles), C. tropicalis FP (triangle), and Rhodotorula sp. G10.2 (diamond); Open symbols: xylose; Full symbols: ethanol. (B) Consumption of xylose and biomass by P. guilliermondii G1.2 (squares half up) and G4.2 (circle half right), C. oleophila G10.1 (star), M. koreensis G18 (diamond half left), R. mucilaginosa G7.2 (cross).

Table 2 – Xylitol productivity by C. tropicalis FP strain. Fermentation realized in a basal medium with xylose (100 g L\(^{-1}\)) and yeast extract (10 g L\(^{-1}\)) at 30 °C in 120 h.

<table>
<thead>
<tr>
<th>Parameters evaluated</th>
<th>Xylitol</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E2</td>
<td>S4</td>
</tr>
<tr>
<td>Sugar consumed (g L(^{-1}))</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Production (g L(^{-1}))</td>
<td>22</td>
<td>15.5</td>
</tr>
<tr>
<td>Yield – (Y_{px}) (g g(^{-1}))</td>
<td>0.22</td>
<td>0.15</td>
</tr>
<tr>
<td>Volumetric productivity – (Q_p) (g L(^{-1}) h(^{-1}))</td>
<td>0.18</td>
<td>0.12</td>
</tr>
<tr>
<td>Conversion efficiency – (\eta) (%)(^a)</td>
<td>24</td>
<td>16</td>
</tr>
</tbody>
</table>

\(^a\) Theoretical yield for xylitol production defined at 0.9 g g\(^{-1}\)^{30} and theoretical yield for ethanol production defined at 0.51 g g\(^{-1}\)^{11}.
Discussion

In general, the yeasts strains presented xylose assimilation but low ethanol production. C. tropicalis S4 produced the highest amount of ethanol (6 g L⁻¹), with a yield of 0.1 g g⁻¹. This strain showed good sugar consumption and the species is not among the most promising yeasts for ethanol production. In fact, the literature shows that the most promising species for ethanol production are *Scheffersomyces stipitis*, S. shehatae and *Pachysolen tannophilus*, while C. tropicalis is related to xylitol production.

The strains *C. oleophila* G10.1, *M. koreensis* G18 and *P. guilliermondii* G4.2 showed smaller yields of biomass production than *P. guilliermondii* G1.2 and *R. mucilaginosa* G7.2. This sugar consumption with minor growth suggests the production of metabolites which were not ethanol or xylitol. Thus, these strains need further investigation about alternative compounds like organic acids.

*P. guilliermondii* is frequently cited for production of xylitol and/or ethanol, but *C. oleophila* and *M. koreensis* have not been reported in the literature for the fermentation of hydrolysate lignocellulosic material. *C. oleophila* has been reported as an agent of biologic control due to its ability to suppress the growth of some fungi that cause fruit spoilage like *Botrytis cinerea* and *Penicillium digitatum*.

*M. koreensis* was described more recently and was reported to be a carbonyl reductase and an L-talitol producer.

The specie *A. pullulans*, a yeast-like, is able to assimilate glucose and xylose but is reported as unable to ferment glucose. This feature makes this yeast interesting in a possible co-cultivation with *S. cerevisiae*. Furthermore, it is reported as being producer of high xylanase activity allowing the hydrolysis of derived from hemicellulose in the production of ethanol from lignocellulosic material.

The strain *C. tropicalis* S4 showed slower xylose assimilation but consumed all the sugar of the medium in 120 h and produced the most ethanol. Furthermore, the decrease in the ethanol in the fermentation media over the course of time indicates that, perhaps, it is able to assimilate the ethanol, which would justify the slow xylose utilization.

Under the conditions tested, *C. tropicalis* FP consumed all the xylose. Nevertheless, it presented a low yield for both ethanol and xylitol. Also, during the fermentation process, the strain did not present a large increase in the biomass (maximum biomass was 7 g L⁻¹), that suggests the presence of other coproducts. Meanwhile, *C. tropicalis* E2 presented high xylose assimilation with a higher yield for xylitol than for ethanol, showing its promise for research into xylitol production.

The amount of xylitol produced by *C. tropicalis* strains shows the importance of the individual study of the characteristics of each strain, since differences may occur in the production of compounds, although belonging to the same species. While the strain E2 produced a small quantity of ethanol and a larger quantity of xylitol, the strain FP produced the same quantity of both compounds.

The ethanol and xylitol production is strongly influenced by the fermentation conditions such as pH, temperature, oxygen and nutrients which were not studied in this work. Maybe productivity could be increased in different fermentation conditions, mainly controlling the oxygen supply.

The missing of ethanol production from xylose by the most of yeasts is marked by a redox imbalance that occurs between cofactors NAD and NADP during the formation of xylitol and xylulose. The xylose reductase enzyme reduces D-xyllose to xylitol using NADPH, whereas xylitol dehydrogenase requires NAD⁺ mainly, which hampers the recycling of cofactors. This imbalance causes xylitol accumulation in the medium and the pathway does not proceed to produce ethanol. The recycling of NAD⁺ can be relieved through respiratory chain. However, depending on the oxygen level, cell growth may occur instead of ethanol production.

Xylitol is industrially interesting for its applicability as a sweetener for diabetics and is utilized in the food, pharmaceutical, oral health and cosmetics industries. In addition, its production from several lignocellulosic materials, such as corn cob and cotton stalk, has been tested. Furthermore, to increase the xylitol yield from lignocellulosic materials, some studies looked at strategies such as hydrolysate detoxification or yeast adaptation, cell immobilization and varying the process conditions.

In conclusion, the assimilating yeasts are common in the environment but the understanding of the absorption and assimilation of the sugar is needed so that biotechnological utilization of this pathway is possible. Our results showed the importance of the individual study of each strain, since they have different characteristics concerning response to external factors, even if the strains belong to the same species. The study of the production of alternative compounds to ethanol is important because the application of these strains in industrial processes has not been well studied.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

The authors would like to thanks Fundação de Amparo à Pesquisa do Estado de São Paulo (Proc n° 2010/12624-0), Conselho Nacional de Desenvolvimento Científico e Tecnológico and Coordenação de Aperfeiçoamento de Pessoal em Nível Superior for their financial support and Daniela Correia de Oliveira Lisboa for the technician support.

References


