Phenylalanine ammonia lyase: new insights from Piperaceae species

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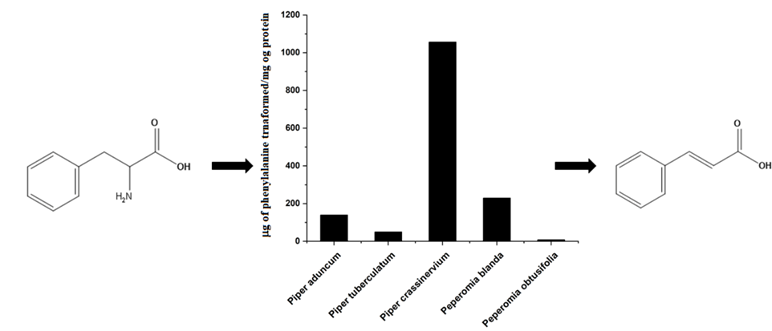
1. *Peperomia*

2. *Piper*

3. phenylalanine ammonia lyase

4. phenylpropanoid derivatives

5. Thermal stability



**ABSTRACT:** The enzyme PAL (phenylalanine ammonia lyase) mediates the key entry point to the general phenylpropanoid pathway, which is involved in the lignification process and in the formation of a myriad of secondary compounds in plants that show a variety of biological activities. Soluble fractions containing PAL extracted from *Piper* and *Peperomia* species had the optimal catalytic activity analyzed by statistical design model. This analysis revealed that the best conversion of *L*-phenylalanine to *trans*-cinnamic acid was pH 9.3 and 58 °C after 25 h, corroborating interesting thermal stability. Additionally, the pre-purification of PAL using ammonium sulfate precipitation (25-55%) increased its specific activity, approximately 133% in *P. aduncum* and more than 900% in *P. crassinervium*. The content of lignin was higher for

*P. tuberculatum* (25.71%), while only a small amount of lignin was observed in *Peperomia blanda* (11.95%). It is interesting to note that *Peperomia* plants are succulent and without significant amounts of lignin. However, the phenylpropanoid biosynthetic pathway is apparently addressed to produce predominantly tetrahydrofuran lignans with biological interest.

1. **1. Introduction**
2. Phenylalanine ammonia lyase (PAL, EC 4.3.1.24) ([Toscano *et al.*, 2018](#ref86)) plays a crucial role at the interface between primary and secondary metabolism in plants - it catalyzes the *trans-*deamination of *L*-phenylalanineto *trans*-cinnamic acid, which is the first step in thegeneral phenylpropanoid pathway ([Huang *et al.*, 2020](#ref35); [Levy *et al.*, 2018](#ref45); [Machado *et al.*, 2013](#ref50)). This enzyme has an important role in plant growth since it is required to produce the macromolecule lignin in plants, which has a primary structural function and acts as physical and chemical barriers against pathogens ([Gutiérrez-Quequezana *et al.*, 2020](#ref31); [Vanholme *et al.*, 2019](#ref88); [You *et al.*, 2020](#ref95)).
3. The phenylpropanoid pathway, one of the most important secondary metabolism pathways, leads to the biosynthesis of a wide range of secondary metabolites, such as flavonoids, isoflavonoids, anthocyanins ([Dong *et al.*, 2016](#ref19); [Mota *et al.*, 2011](#ref60); [Prabpree *et al.*, 2018](#ref67)), phenylalanine derivatives ([Batista Junior *et al.*, 2009](#ref09); [2010](#ref10); [Batista *et al.*, 2012](#ref06); [Felippe *et al.*, 2012](#ref25); [Moraes and Kato, 2021](#ref57); [Ramos *et al.*, 2013](#ref68); [Yoshida *et al.*, 2018](#ref94); [You *et al.*, 2020](#ref95)), phytoalexins ([Dong *et al.*, 2016](#ref19)), chromenes ([Batista *et al.*, 2017](#ref07); [Kitamura *et al.*, 2006](#ref41); [Lago *et al.*, 2004](#ref43); [Morandim *et al.*, 2005](#ref58); [Salazar *et al.*, 2005](#ref74); [Souza *et al.*, 2019](#ref84)), chromanes ([Batista Junior *et al.*, 2012](#ref11); [El Babili *et al.*, 2021](#ref23)), amides ([García-Huertas *et al.*, 2018](#ref30); [Lopes *et al.*, 2007](#ref47); [López *et al.*, 2010](#ref48); [Mota *et al.*, 2009](#ref59)), monolignols ([Macêdo](#ref49) *[et al.](#ref49)*[, 2020](#ref49)), coumarins ([Banu *et al.*, 2019](#ref05)), and lignans ([Dong *et al.*, 2016](#ref19)) ([Fig. 1](#fig01)).

Mapa com linhas pretas em fundo branco

Descrição gerada automaticamente

**Figure 1**. Phenylpropanoid pathways involved in the formation of lignin.

1. **Source:** Adapted from [Vanholme *et al*. (2019)](#ref88).
2. In the lignin pathway, three principal enzymes are involved: phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase, and 4-coumarate: CoA ligase ([Banu *et al.*, 2019](#ref05); [Vanholme *et al.*, 2019](#ref88)). In the lignin biosynthesis in plants, PAL is the primer enzyme that mediates the formation of the cinnamic acid, which is the first product in the general phenylpropanoid pathway ([Dong *et al.*, 2016](#ref19); [Felippe *et al.*, 2012](#ref25); [Klejdus *et al.*, 2013](#ref42); [Machado *et al.*, 2013](#ref50)). A series of enzymes are required to produce the cinnamyl alcohols required for the radical reaction: hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase, hydroxycinnamoyl CoA:quinate hydroxycinnamoyl transferase, *p*-coumaroyl shikimate 3’-hydroxylase, and ferulate (coniferaldehyde) 5-hydroxylase ([Kamdee](#ref38) *[et al.](#ref38)*[, 2014](#ref38)). These units are mainly oxidized by the action of peroxidases and laccases. Subsequently, the lignin phenoxyl radicals react to form lignin ([A. Silva *et al.*, 2019a](#ref81); [Veronico *et al.*, 2018](#ref89); [Weiss *et al.*, 2020](#ref92)).
3. In this context, in the plant biomass, lignin (whose content depends on plant species and tissues) is associated with cellulose and hemicellulose and prevents biological degradation of these materials, as well as providing mechanical support for plant tissues. Therefore, lignin confers strength and rigidity to the cellulose fiber’s assembly ([Gao *et al.*, 2019](#ref29)). After cellulose, lignin is the second most abundant organic compound in plants, representing approximately 30% of the organic carbon in the biosphere ([Kamdee](#ref38) *[et al.](#ref38)*[, 2014](#ref38)). From a functional point of view, lignin imparts strength to cell walls, facilitates water transport, and prevents degradation of wall polysaccharides, thereby acting as a major defense line against pathogens, insects, and other herbivores ([Tronchet](#ref87) *[et al.](#ref87)*[, 2010](#ref87)).
4. However, the genetic manipulation of the enzymatic routes, e.g., the PAL enzyme production, may favor the optimization of the separation of lignin from the cellulose pulp. This causes a reduction in the cost and pollution during the lignocellulosic processes, once the lignification is controlled by many enzymes including PAL ([Hamedan *et al.*, 2019](#ref32); [Liu *et al.*, 2019](#ref46); [Toscano *et al.*, 2018](#ref86)). Therefore, by reducing the PAL activity, it is possible to minimize the lignin content resulting in weak stem physical strength ([Hussain *et al.*, 2019](#ref36)).
5. This enzyme is mainly responsible for the biosynthesis of many activity compounds. Previous studies showed that these classes of secondary metabolites are among the most potent biologically active compounds found in *Piper* and *Peperomia* species, mainly species of the Piperaceae ([Alves *et al.*, 2019](#ref01); [Kato and Furlan, 2007](#ref39)). Species of this family present the following activities: antioxidant ([Regasini](#ref70) *[et al.](#ref70)*[, 2008](#ref70); [J. Silva *et al.*, 2011](#ref79); [N. Silva et al., 2019b](#ref82); [Yamaguchi *et al.*, 2006](#ref93)), anxiolytic, antiplatelet ([Sant’Ana](#ref75) *[et al.](#ref75)*[, 2020](#ref75)), antitumoral ([Cortez *et al.*, 2017](#ref17); [H. Silva *et al.*, 2018](#ref80)), cytotoxic ([Ferreira *et al.*, 2014](#ref26); [Freitas *et al.*, 2014](#ref28); [Maleck *et al.*, 2014](#ref51)), trypanocidal ([Batista Junior *et al.*, 2008](#ref08); [Felippe *et al.*, 2008](#ref24); [García-Huertas *et al.*, 2018](#ref30); [Martins *et al.*, 2003](#ref53); [Mota *et al.*, 2009](#ref59); [Regasini *et al.*, 2009](#ref71)), hypertension ([Durant-Archibold *et al.*, 2018](#ref21)), leishmanicidal ([Araújo-Vilges *et al.*, 2017](#ref04); [Macêdo *et al.*, 2020](#ref49); [Neves *et al.*, 2019](#ref63); [Oliveira *et al.*, 2012](#ref64); [M. Silva *et al.*, 2021](#ref83)), insecticidal, antifungal, bactericidal, acaricidal, molluscicidal ([Corral *et al.*, 2018](#ref16); [Danelutte *et al.*, 2003](#ref18); [Ferreira *et al.*, 2014](#ref26); [Lago *et al.*, 2004](#ref43); [2007](#ref44); [Marques *et al.*, 2010](#ref52); [Miranda *et al.*, 2003](#ref55); [Navickiene *et al.*, 2000](#ref61); [2003](#ref62); [Ramos *et al.*, 2020](#ref69); [Reigada *et al.*, 2007](#ref72); [Santos *et al.*, 2013](#ref76); [R. Silva *et al.*, 2002](#ref78)), anti-inflammatory ([Arunachalam *et al.*, 2020](#ref03)), larvicidal ([Mgbeahuruike](#ref54) *[et al.](#ref54)*[, 2017](#ref54); [Pereira Filho *et al.*, 2021](#ref65); [Pinheiro *et al.*, 2011](#ref66); [N. Silva](#ref82) *[et al.](#ref82)*[,](#ref82)
6. [2019b](#ref82)), antiherbivore ([Jeffrey *et al.*, 2014](#ref37)), and antimalarial ([Campelo](#ref14) *[et al.](#ref14)*[, 2018](#ref14); [Moraes *et al.*, 2013](#ref56); [H. Silva *et al.*, 2018](#ref80)). Additionally, studies on the applications of the PAL enzyme have reported its use to treat diseases, such as phenylketonuria ([Burton *et al.*, 2020](#ref13); [Hartnett *et al.*, 2013](#ref34); [Levy *et al.*, 2018](#ref45)). This disease is a genetic mutation that inactivates the phenylalanine-4-hydroxylase ([Burton *et al.*, 2020](#ref13); [Levy *et al.*, 2018](#ref45); [Sarkissian *et al.*, 2011](#ref77); [Vogel *et al.*, 2013](#ref90)), resulting in augmented levels of *L*-phenylalanine in the bloodstream by the inability to metabolize *L*-phenylalanine. In excess, phenylalanine itself shows a neurotoxic effect. The lack of treatment at birth leads to severe and irreversible mental retardation ([Donlon *et al.*, 2004](#ref20)). Therefore, PAL has gained considerable significance in several clinical and industrial applications in the past three decades. In the clinical field, physicians have used PAL to determine the serum level of *L*-phenylalanine, monitoring patients with phenylketonuria ([Burton *et al.*, 2020](#ref13); [Watanabe *et al.*, 1992](#ref91)) and then to prepare low phenylalanine diets. Enzyme substitution therapy with a recombinant form of PAL successfully converts the excess *L*-phenylalanine into harmless metabolites, *trans*-cinnamic acid, and ammonia, which can be excreted in the urine ([Rocha *et al.*, 2021](#ref73)).
7. A major industrial application of PAL concerns the production of *L*-phenylalanine from *trans*-cinnamic acid by reversing the physiological reaction ([Burton *et al.*, 2020](#ref13); [Hamilton *et al.*, 1985](#ref33)). The increasing demand for *L*-phenylalanine as a precursor of aspartame, a non-calorific sweetener (*L*-phenylalanyl-*L*-aspartyl methyl ester), has resulted in an industrial enzymatic process based on *Rhodotorula* cells immobilized in vermiculite, developed by Genex Corporation in the UK ([Choudhary and Lee, 2018](#ref15); [Finkelman and Yang, 1985](#ref27)).
8. Herein, the results of the expression of PAL in leaves of five Piperaceae species are reported. The results provide insights into the PAL catalytic activities in these species, which are rich sources of the bioactive phenylpropanoid derivatives and open new opportunities to enhance the production of such class of metabolites. Furthermore, this study also provides relevant results about the activity effect on its concentration, which represents new opportunities for the industrial and agricultural applications, especially favored by its thermal stability.
9. **2. Experimental**
10. ***2.1 Plant material***
11. Leavesof *Piper aduncum* L. and *Peperomia obtusifolia* (L.) A. Dietr. were collected at the Institute of Chemistry – UNESP, Araraquara–SP, Brazil (-21.806602382914665, -48.19284372352492). These specimens were identified by Dr. I. Cordeiro (Institute of Botany, SP). The voucher specimens (Cordeiro-PA0 and Kato-070) were deposited in the Herbarium at the Institute of Biosciences – USP, São Paulo-SP, Brazil.
12. Leaves of *Piper tuberculatum* Jacq. and *Piper crassinervium* Kunth were collected at the Institute of Chemistry - UNESP), Araraquara-SP, Brazil (-21.806602382914665, -48.19284372352492), and identified by Dr. G. E. D. Paredes (Universidad Nacional Pedro Ruiz Gallo, Peru). The voucher specimens (Kato-163 and Kato-0084) were deposited in the Herbarium at the Institute of Botany, São Paulo–SP, Brazil. Leaves of *Peperomia blanda* (Jacq.) were collected at Reserva da Ripasa, Ibaté–SP, Brazil (-22.237934727962543, -47.81789620915089) and identiﬁed by Dr. Elsie Franklin Guimarães (JBRJ). The voucher specimen (Kato-547) was deposited in the Herbarium at the Institute of Biosciences, USP, São Paulo–SP, Brazil.
13. ***2.2 Reagents and standards***
14. Methanol (HPLC-grade) was purchased from Mallinckrodt (Baker, Xalostoc, Mexico); ascorbic acid, ethylenediamine tetraacetic acid (EDTA), sucrose, polyvinylpolypyrrolidone (PVPP), dithiothreitol (DTT), *L*-phenylalanine, cinnamic acid, boric acid, sodium borate, hydrochloric acid (HCl), and ammonium sulfate were acquired from Sigma-Aldrich (St. Louis, MO, USA). Nanopure water (> 18 M cm) was produced using a Millipore (Bedford, MA, USA) purifier.

***2.3 Enzyme Extraction***

1. Fresh leaves of *Piper aduncum*, *P. tuberculatum*, *P. crassinervium*, as well *Peperomia blanda* and *P. obtusifolia* (25 g) were ground separately using liquid nitrogen. Then, 100 mL of borate buffer 0.1 M (pH 8.8) containing PVPP (10% w/w), sucrose (0.25 M), ascorbic acid (40 mM), EDTA (1 mM), and DTT (5 mM) were added. The suspensions were stirred for 15 min at 0 °C, filtered through cheesecloth, and centrifuged for 20 min at 4 °C and 11,950 g.

***2.4 Enzymatic assay***

1. The enzymatic assays were carried out by adding 250 µL of borate buffer 0.1 M, 125 µL of enzyme solution (0.2 mg/mL), and 150 µL of *L*-phenylalanine (10 mM) to Eppendorf tubes. The times (1, 6, 13, 20, and 25 h), temperatures (28, 35, 45, 55, and 62 ºC) and pH values (7.8, 8.2, 8.8, 9.4, and 9.8) were modified as suggested by the statistical design to optimize the process ([Tab. 1](#tab01)). All the reactions were performed under agitation and terminated by adding 25 µL of HCl (6 M), in duplicate assays ([Toscano *et al.*, 2018](#ref86)). The solutions were stirred, neutralized, and then centrifugated at 7,000 g for 1 min, and the supernatants were analyzed by HPLC.

**Table 1.** Experimental conditions used to optimize the activity of the PAL (coded and non-coded variables).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Coded variables | | | Non-coded variables | | |
| **Time (min)** | **pH** | **T (ºC)** | **Time (min)** | **pH** | **T (ºC)** |
| +1 | -1 | +1 | 20 | 8.20 | 55 |
| +1 | +1 | +1 | 20 | 9.40 | 55 |
| 0 | 0 | 0 | 13 | 8.80 | 45 |
| 0 | + | 0 | 13 | 9.80 | 45 |
| 0 | 0 | + | 13 | 8.80 | 62 |
| - | 0 | 0 | 1 | 8.80 | 45 |
| 0 | - | 0 | 13 | 7.80 | 45 |
| +1 | -1 | -1 | 20 | 8.20 | 35 |
| -1 | +1 | -1 | 6 | 9.40 | 35 |
| 0 | 0 | - | 13 | 8.80 | 28 |
| -1 | -1 | -1 | 6 | 8.20 | 35 |
| -1 | -1 | +1 | 6 | 8.20 | 55 |
| +1 | +1 | -1 | 20 | 9.40 | 35 |
| -1 | +1 | +1 | 6 | 9.40 | 55 |
| + | 0 | 0 | 25 | 8.80 | 45 |
| 0 | 0 | 0 | 13 | 8.80 | 45 |

1. HPLC analyses were conducted on a Shimadzu pump model LC-6AD, with an SPD-M20A diode array ultraviolet detector (HPLC-DAD), SIL-10AF automatic injector, and CBM-20A controller. Separation was achieved on a Phenomenex C18 reverse-phase column (25 cm × 4.6 mm, 5 μm) in the isocratic mode, using MeOH/H2O (1:1, with 0.1% HOAc) at a flow of 1.0 mL min–1 as the mobile phase. The formation of *trans*-cinnamic acid was monitored at a wavelength of 275 nm.
2. A standard RSM (Response Surface Methodology) design, known as CCRD (Central Composite Rotational Design), was used to model and analyze the data. The Analysis of Variance (ANOVA) using the Statistic Program 12.0 (StatSoft Inc., 2014, Oklahoma, USA) was used to process the results. The significance of the main factors and their interactions were assessed by the F-test method with a confidence level of 95%. The response surface methodology and a mathematical–statistical tool were employed to model the *L*-phenylalanine conversion to *trans*-cinnamic acid using the temperature, pH, and reaction time as variables. The Response desirability profiling option, which is given by the geometric mean of all individual desirability ([Eq. 1](#eq01)), was applied to determine the optimum reaction condition for the PAL enzyme.
3. (1)
4. where, *d* describes the individual desirability and *m* the number of parameters analyzed.
5. The results of each CCRD were analyzed with the aid of the Statistic Program Software. The data was fitted to a second-order polynomial equation ([Eq. 2](#eq02)).
6. Y=βo +∑βjxj +∑βjjxj2 +∑βjkxjxk (2)
7. ***2.5 Enzyme pre-purification***
8. After determining the optimal reaction condition, new enzymatic extracts were obtained from the same plant species using the experimental procedures described above. In this case, 100 g of plant material and 400 mL of buffer were used. The protein extracts containing PAL were fractionated by ammonium sulfate saturation at 0-25, 25-55, and 55-80%. Each addition of ammonium sulfate was followed by agitation for 30 min at 4 °C and then centrifugated at 16,300 g for 30 min. The pellets were stored at -80 ºC until use. The pellets were re-suspended in 3 mL of 0.1 mol L–1 borate buffer and submitted to enzymatic assays. The amount of added ammonium sulfate to achieve the desired saturation was determined as described ([Englard and Seifter, 1990](#ref22)). Protein was determined by the modified Bradford method, using bovine serum albumin (BSA) as standard.

***2.6 Determination of lignin content***

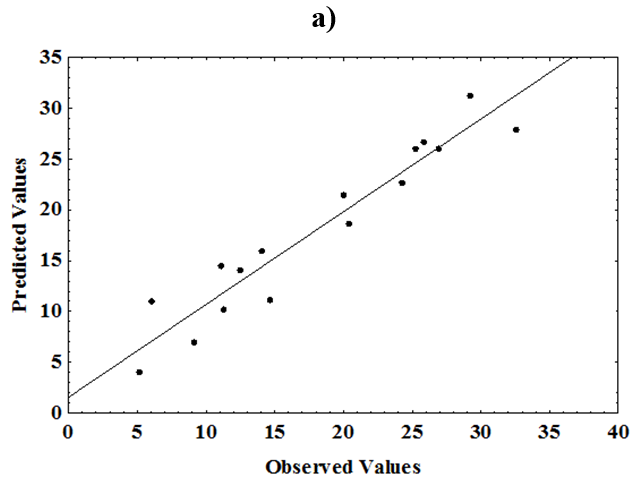
Leaves of the target plant species were dried at (70 ± 3) ºC for 72 h, knife-ground in order to pass through a 1-mm sieve and be analyzed. The lignin contents were determined using the TAPPI test method ([Begović](#ref12) *[et al.](#ref12)*[, 2018](#ref12); [TAPPI, 1979](#ref85)).

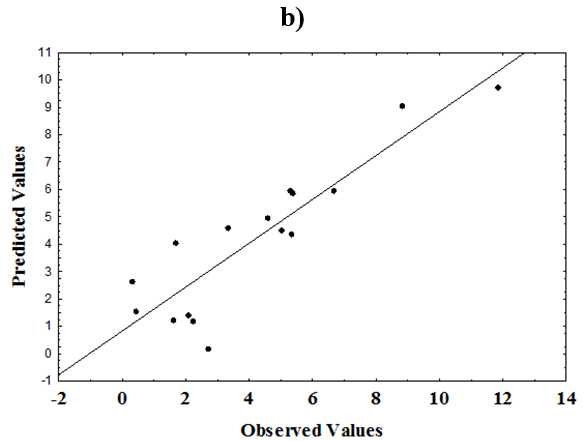
1. **3. Results and Discussions**
2. The enzyme phenylalanine ammonia lyase (PAL) is the initial enzyme involved in the *trans*-deamination of *L*-phenylalanine leading to the formation of *trans*-cinnamic acid. PAL is a regulatory enzyme involved in the phenylpropanoid pathway of several plants and provides insights to control the production of these secondary metabolites ([Akbarian](#ref02) *[et al.](#ref02)*[, 2021](#ref02); [Kawatra *et al.*, 2020](#ref40)).
3. The first step of this work consisted in optimizing the reaction conditions for the measurement of the PAL activity. Since several Piperaceae species belonging to *Piper* (*P. aduncum*, *P. tuberculatum*, *P. crassinervium*) and *Peperomia* (*P. blanda* and *P. obtusifolia*) species have been characterized to produce cinnamic acid derivatives, they were considered as potential sources of the PAL enzyme. *Peperomia obtusifolia*, which shows chromanes as the major metabolites, was selected as no model plant for PAL.
4. Another important physiological aspect of these plant species is their relatively easy propagation and fast growth. Therefore, crude enzymatic extracts from the five Piperaceae species were obtained, and the relative activities of PAL determined, monitoring the conversion of phenylalanine into cinnamic acid by HPLC. Then, the reaction conditions were optimized regarding temperature, pH, and reaction time for each *Piper* and *Peperomia* species ([Tab. 1](#tab01) and [2](#tab02)). The results showed that the increase in reaction time also increases the *trans*-cinnamic acid conversion. The same occurred regarding pH and temperature, with a great reaction observed at around 50 °C and pH 8.8. Therefore, in order to investigate the influence of all process variables and determine the optimal PAL activity, a statistical study was performed.

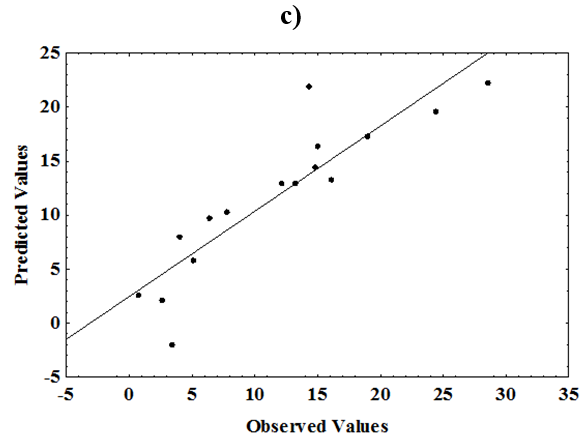
The Statistic 12.0 Program helped to analyze the data and to optimize the operation conditions for the PAL enzyme. The data were evaluated using the linear and quadratic terms model. The relationship between the proposed model and the theoretical data was outlined and the results are shown in [Fig. 2](#fig02a). The results evidenced the normality of the residues, since all the points were close to the expected values.

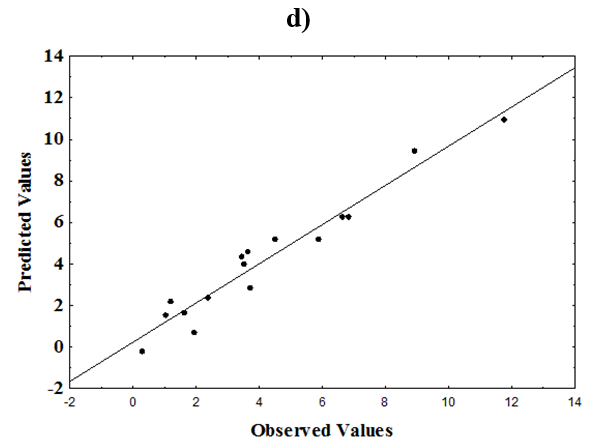
**Table 2.** Reaction conditions for PAL and conversion % of *L*-phenylalanine into cinnamic acid for each Piperaceae species.

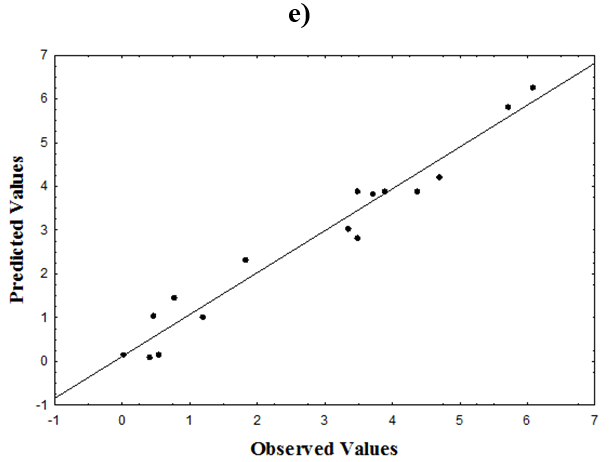
|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Time (min) | pH | T (ºC) | *P. aduncum* | *P. tuberculatum* | *P. crassinervium* | *P. blanda* | *P. obtusifolia* |
| **(a)** | **(b)** | **(c)** | **(d)** | **(e)** |
| 13 | 8.80 | 28 | 14.06 | 2.23 | 6.38 | 1.19 | 0.77 |
| 6 | 8.20 | 35 | 9.14 | 1.62 | 3.46 | 1.05 | 0.53 |
| 20 | 8.20 | 35 | 12.46 | 4.57 | 7.74 | 3.70 | 3.49 |
| 6 | 9.40 | 35 | 14.65 | 2.06 | 5.07 | 1.96 | 0.41 |
| 20 | 9.40 | 35 | 25.81 | 1.68 | 18.96 | 6.64 | 3.88 |
| 13 | 7.80 | 45 | 5.18 | 0.42 | 2.58 | 0.28 | 0.47 |
| 1 | 8.80 | 45 | 6.08 | 0.32 | 0.79 | 2.39 | 0.01 |
| 13 | 8.80 | 45 | 26.93 | 6.66 | 13.20 | 5.90 | 4.36 |
| 13 | 8.80 | 45 | 25.25 | 5.28 | 12.16 | 4.51 | 3.48 |
| 25 | 8.80 | 45 | 32.58 | 11.85 | 24.43 | 8.93 | 5.72 |
| 13 | 9.80 | 45 | 20.02 | 5.32 | 16.08 | 3.62 | 3.34 |
| 6 | 8.20 | 55 | 11.25 | 2.71 | 4.04 | 1.64 | 1.20 |
| 20 | 8.20 | 55 | 11.11 | 5.36 | 14.96 | 3.43 | 3.71 |
| 20 | 9.40 | 55 | 29.17 | 8.81 | 14.27 | 11.74 | 6.07 |
| 6 | 9.40 | 55 | 20.39 | 5.01 | 14.80 | 3.50 | 1.83 |
| 13 | 8.80 | 62 | 24.29 | 3.35 | 28.59 | 6.82 | 4.69 |





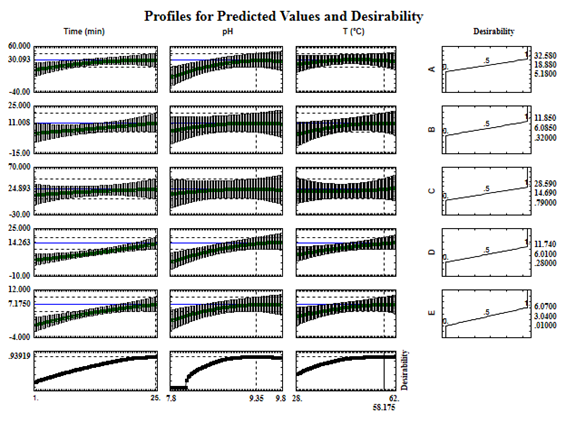






**Figure 2**. A comparative plot between the experimental and the predicted yield as a function of time (h), temperature (ºC), and pH for the enzymatic extracts from **(a)** *P. aduncum*,**(b)** *P. tuberculatum*,**(c)** *P. crassinervium*,**(d)** *Peperomia blanda*,and **(e)** *P. obtusifolia*.

1. After adjusting the model, the data were analyzed using the desirability function. After 20 h of reaction, the conversion of *L*-phenylalanine into *trans*-cinnamic acid did not increase significantly, and it proved to be the best reaction time. The analysis of its thermal resistance showed a maximum performance after 40 ºC, with an even larger increase above 49 ºC. The optimal pH for the reaction was 9.4 ([Fig. 3](#fig03)).
2. The plot contour surface obtained for the enzymatic extracts illustrates the results ([Fig. 4](#fig04)). Basic pH values (9.0 to 9.8) afforded the best results of *L*-phenylalanine conversion into *trans*-cinnamic acid. The thermostability of the enzyme was also evident: optimal reaction occurred at temperatures above 40 ºC. The conversion persisted even after 20 h despite the low rate. The previously described statistical procedure provided the Equations, which showed the r2 value at a level of confidence of 95% for *P. aduncum* (A), *P. tuberculatum* (B), *P. crassinervium* (C), *P. blanda* (D), and *P. obtusifolia* (E) ([Tab. 3](#tab03)).



1. **Figure 3**. PAL activity from Piperaceae species taking independent and dependent variables into account: (A) *P. aduncum*,(B) *P. tuberculatum*,(C) *P. crassinervium*,(D) *Peperomia blanda*,and (E) *P. obtusifolia.*
2. Gráfico

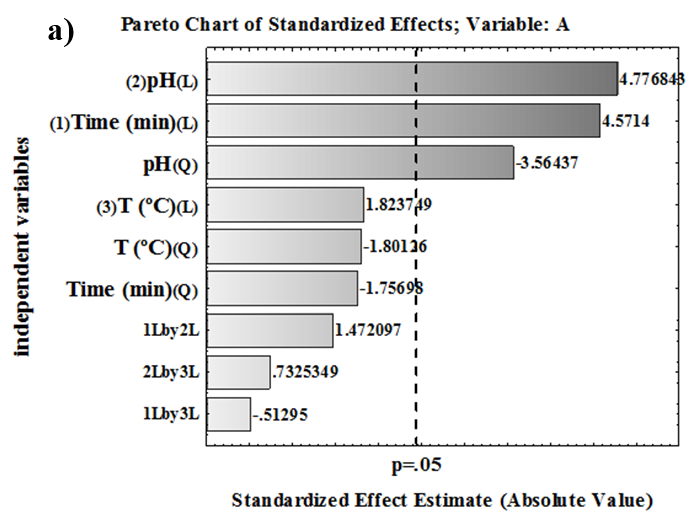
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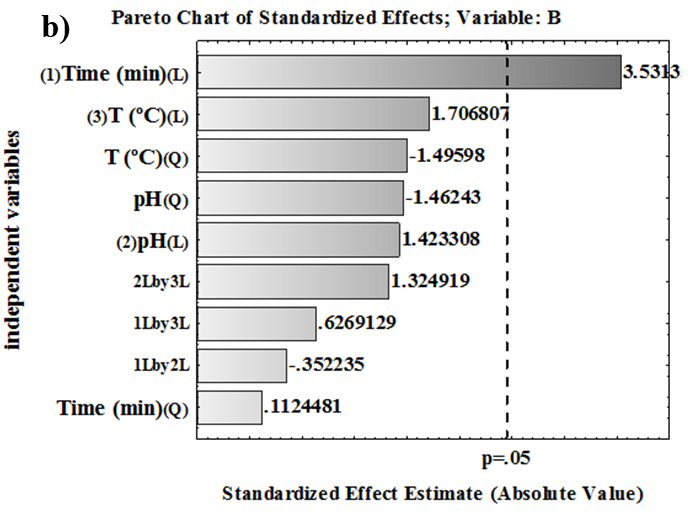
**Figure 4**. Contour surface graphs for the activity of the PAL enzyme using Response desirability surface/Contours.

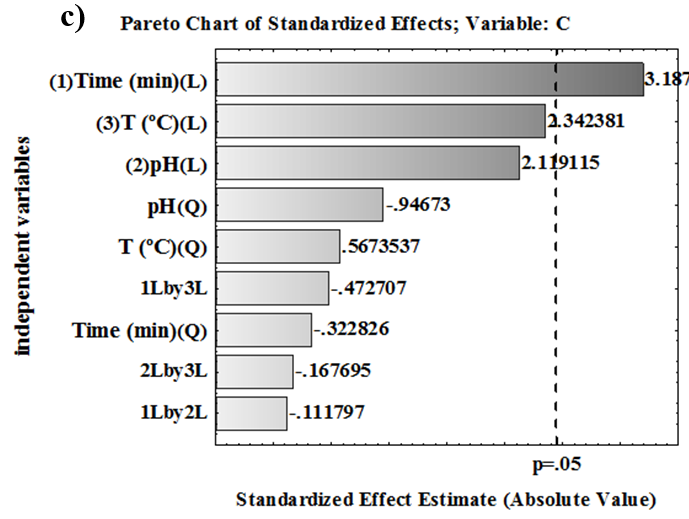
**Table 3.** Coefficients of the Fitted Equations for the Construction of the Surface Response Plots.

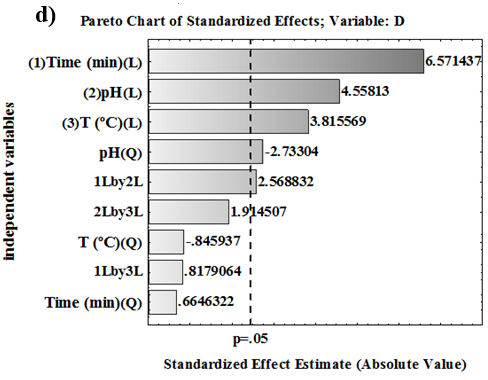
|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Polynomial equation: a0 + a1X1 + a2X2 + a3X3 + a4X1X2 + a5X1X3 + a6X2X3 + a7X12 + a8X22 + a9X32 | | | | | | | | | | | |
|  |  | **Linear parameters** | | | | | | | **Quadratic Parameters** | | |
|  | **R2** | **a0** | **a1** | **a2** | **a3** | **a4** | **a5** | **a6** | **a7** | **a8** | **a9** |
| a | 0.91284 | -1038.26 | -2.02 | 229.43 | 0.92 | 0.50 | -0.01 | 0.17 | -0.05 | -13.35 | -0.02 |
| b | 0.80069 | -202.386 | 0.514 | 46.986 | -0.539 | -0.065\*t | 0.007 | 0.171 | 0.002 | -2.978 | -0.011 |
| c | 0.78952 | -476.660 | 2.136 | 99.124 | 0.092 | -0.055 | -0.014 | -0.058 | -0.012 | -5.127 | 0.011 |
| d | 0.94398 | -176.934 | -2.275 | 45.454 | -0.814 | 0.252 | 0.05 | 0.132 | 0.005 | -2.969 | -0.003 |
| e | 0.95724 | -134.596 | -0.210 | 30.532 | -0.097 | 0.067 | 0.001 | 0.057 | -0.006 | -1.873 | -0.004 |

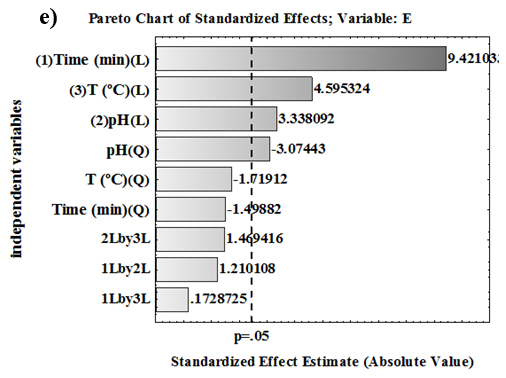
1. Besides checking the fit of the model and optimizing the operation conditions, the variables that most influenced the PAL activity of the different plant extracts were investigated ([Fig. 5](#fig05a)).





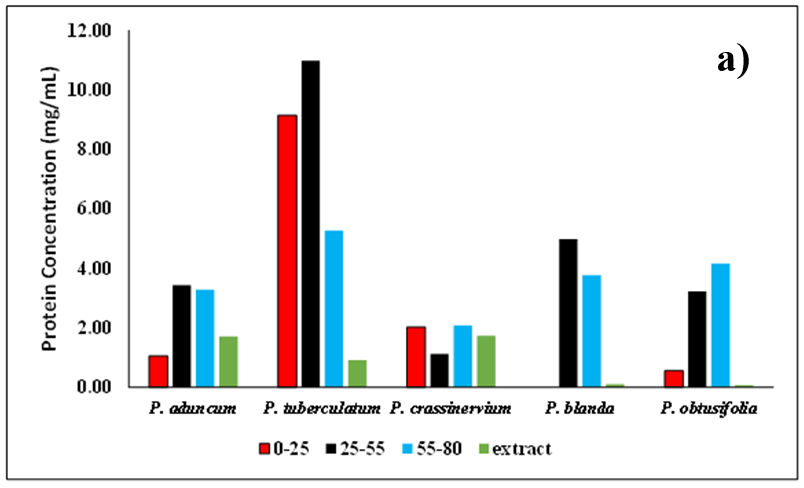


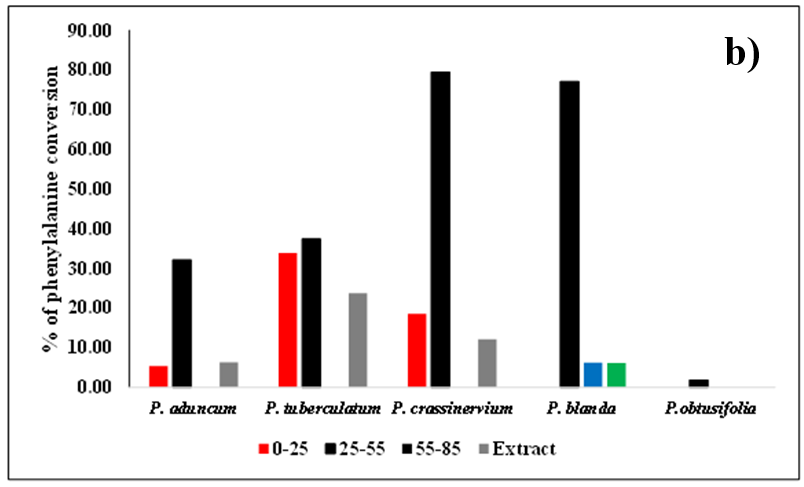


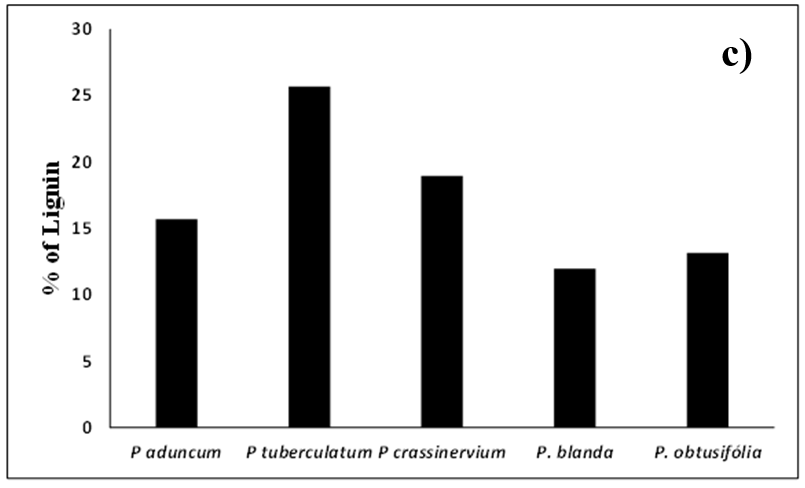


**Figure 5.** Analysis of the variables that most affect the activity of the PAL enzyme.

1. The variables that most influenced the process were pH and time. Regarding temperature, PAL showed the optimum activity at 58 °C in all plant species, showing thermal stability. These results corroborate their possible application in biotechnological processes.
2. Therefore, after determining the PAL optimum conditions, an ammonium sulfate precipitation was performed, and fractions of 0-25%, 25-55%, and 55-80 were obtained. The protein content of the enzymatic extracts obtained from leaves was determined and revealed that higher protein concentrations with 25-55% saturation were found in *P. aduncum*, *P. tuberculatum*, and *P. blanda*. However, *P. crassinervium* and *P. obtusifolia* displayed a higher protein content in the fraction with 55-80% saturation ([Fig. 6a](#fig06a)).







**Figure 6.** **(a)** Protein content in each of the fractions obtained after saline precipitation, **(b)** comparison of the degree of phenylalanine conversion to cinnamic acid**,** and **(c)** percentage of lignin.

1. By analyzing the obtained fraction with 25-55% of saturation with ammonium sulfate, it was verified that the specific activity in the *P. aduncum* species increased by approximately 133% ([Tab. 4](#tab04), [Fig. 6b](#fig06b)). Regarding *P. tuberculatum*, the specific activity decreased by 87.5% after the saline precipitation step. In *Peperomia blanda*, it was possible to verify a reduction in specific activity of 72.6%, also observed in *P. tuberculatum*. However, in *P. crassinervium*, the specific activity increased by more than 900% ([Tab. 4](#tab04)), demonstrating to be an excellent source of PAL.
2. **Table 4**. Specific activity of PAL observed in the extracts and fractions after precipitation.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | *P. aduncum* | *P. tuberculatum* | *P. crassinervium* | *P. blanda* | *P. obtusifolia* |
|  | **(a)** | **(b)** | **(c)** | **(d)** | **(e)** |
| **Extract** | 60 | 400 | 100 | 840 | 0 |
| **0-25%** | 80 | 60 | 130 | 0 | 0 |
| **25-55%** | 140 | 50 | 1057 | 230 | 9 |
| **55-85%** | 0 | 0 | 0 | 20 | 0 |

1. Considering the last species studied, *P. obtusifolia*, it was possible to observe a significant increase in the specific activity of the PAL enzyme. However, as the protein concentration was too low ([Fig. 6b](#fig06b)), the use of the species during the attainment of the PAL enzyme in a large scale was not feasible ([Tab. 4](#tab04)).
2. These fractions served as the source of the PAL enzyme in further experimental reactions. HPLC analyses evidenced that most of the desired enzyme, PAL, was concentrated in the fraction with 25-55% saturation, attested to the efficiency of the PAL concentration process. The degree of *L*-phenylalanine conversion to *trans*-cinnamic acid increased considerably in all cases, which facilitated the subsequent purification process ([Fig. 6b](#fig06b)).
3. Starting from the same mass of fresh leaves, the enzymatic extracts from *P. crassinervium, P. tuberculatum,* and *P. blanda* precipitated with 25-55% saturation of ammonium sulfate afforded the highest specific activities of PAL. However, the major secondary metabolites found in *P. obtusifolia* are chromanes. They differ from chromenes of *P. aduncum*, originated from the shikimate pathway and which require the *p*-hydroxybenzoic acid as a precursor. The chromanes are structurally related to the orsellinic acid, which is produced by the polyketide pathway. Therefore, these two distinct biosynthetic routes match with the lower PALactivity for *P. obtusifolia.*
4. In order to better characterize the Piperaceae species with a low level of phenylpropanoids pathway, experiments were conducted to examine the original lignin content in each species using the TAPPI test method ([Fig. 6c](#fig06c)). Data analyses of lignin contents demonstrated that the more rigid the leaves, the higher the lignin content (see data for *P. aduncum*, *P. tuberculatum*, and *P. crassinervium*). A small amount of lignin accumulated in the *P. blanda* species suggested that the biosynthetic pathway operated to produce secondary metabolites such as lignoids.

**4. Conclusions**

1. This work enabled the optimization of the reaction conditions to convert *L*-phenylalanine to *trans*-cinnamic acid catalyzed by the PAL enzyme extracted from five Piperaceae species (*Piper aduncum*, *P. tuberculatum*, *P. crassinervium,* *Peperomia* *blanda* and *P. obtusifolia*). Thenzymee exhibited thermostability, and the best reaction condition was pH 9.3 at a temperature of 58 °C for 25 h. It is important to mention that reaction times longer than 20 h do not give rise to significant improvement in the conversion, which did not justify longer reaction times. Pre-purification of the enzyme by ammonium sulfate precipitation showed that the ideal saturation was 25-55% — it increased the enzyme’s concentration and effectively improved phenylalanine’s conversion to cinnamic acid. Additionally, the thermal stability of the PAL may enable the use of the five studied Piperaceae species in specific green industrial processes.

**Authors’ contribution**

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**Project administration:** Morandim-Giannetti, A. A.; Felippe, L. G.; Furlan, M.

**Resources:** Morandim-Giannetti, A. A.; Felippe, L. G.; Santos, V. A. F. F. M.; Kato, M. J.; Furlan, M.

**Software:** Not applicable.

**Supervision:** Morandim-Giannetti, A. A.; Kato, M. J.; Furlan, M.

**Validation:** Morandim-Giannetti, A. A.

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**Writing – review & editing:** Morandim-Giannetti, A. A.; Furlan, M.

**Data availability statement**

All data generated or analyzed during this study are included in this published article.

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