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Biotransformation of the Proteogenic Amino Acids Phenylalanine, Tyrosine and Tryptophan by *Yarrowia* Species: An Application to the Preparative Synthesis of Natural Phenylacetic Acid

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Abstract: The biotransformation of the aromatic amino acids phenylalanine, tyrosine and tryptophan originates a number of bioactive compounds. Yeasts are the most used microorganisms for the transformation of (L)-phenylalanine into the flavour phenylethanol. Here, we reported a study on the biotransformation of the proteogenic aminoacids phenylalanine, tyrosine and tryptophan by yeast strains belonging to *Yarrowia* genus. We found that the latter microorganisms, in high aerobic conditions, metabolise the aromatic amino acids (L)-phenylalanine and (L)-tyrosine with the almost exclusive formation of phenylacetic acid and 4-hydroxyphenylacetic acid, respectively. Differently, the biotransformation of (L)-tryptophan with *Y. lipolytica*, gave anthranilic acid as the main product. As stated by the European and USA legislations concerning natural flavour production, phenylacetic acid obtained by microbial conversion of phenylalanine of natural origin can be commercialised as a natural flavour. Accordingly, our findings were exploited in a new process, based on the *Yarrowia* strains-mediated biotransformation of natural (L)-phenylalanine, that allows the large-scale preparation of the high-value, natural flavour, phenylacetic acid.

Keywords: phenylacetic acid; natural flavours; biotransformation; *Yarrowia*; biocatalysis; whole-cell process; phenylalanine; tyrosine; tryptophan; anthranilic acid

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1. Introduction

The Ehrlich pathway [1] is the most relevant process of amino acid transformation in yeast, whose catabolism is an important source of bioactive compounds. In particular, the aromatic amino acids phenylalanine, tyrosine and tryptophan are precursors of different classes of compounds of commercial relevance, such as flavours [2–7], phytohormones [8,9], and anti-inflammatory, antiviral or antimicrobial agents [10–12].

According to the Ehrlich pathway, after the initial transamination reaction (Figure 1), the amino acids **1a–c** give the corresponding substituted pyruvic acids **2a–c**, which can be the subject of different biochemical transformations. The reduction of the keto functional group affords aryl lactic acids **3a–c** whereas the decarboxylation reaction leads to the formation of the aldehydes **4a–c**. The latter compounds, usually do not accumulate in yeast cells and are subjected to either reduction or oxidation to afford 2-aryl ethanol **5a–c** or 2-aryl acetic acids **6a–c**.

Phenylalanine transformation is the most important method of production of natural phenylethanol (PE) **5a** [2]. This compound, as well as its derivatives, are widely used as a flavour ingredient [13] ([14], p. 1633) and have been recognised as safe for food flavouring [15], as certified by the Flavor & Extract Manufacturers Association (FEMA) that included phenethyl alcohol, aldehyde, acid, and related acetals and esters in the list of the substances Generally Recognized As Safe (GRAS). In addition, phenylalanine is currently

used as starting material for the synthesis of natural phenylacetic acid (PAA) **6a**. Indeed, although the direct microbial biotransformation of **1a** into **6a** has been exploited in only a few processes [3,4], the main part of the natural PAA is currently produced by a two steps pathway, based on the transformation of **1a** into PE **5a** followed by its oxidation to the corresponding acid by fermentation with *Gluconobacter oxidans* [5–7].

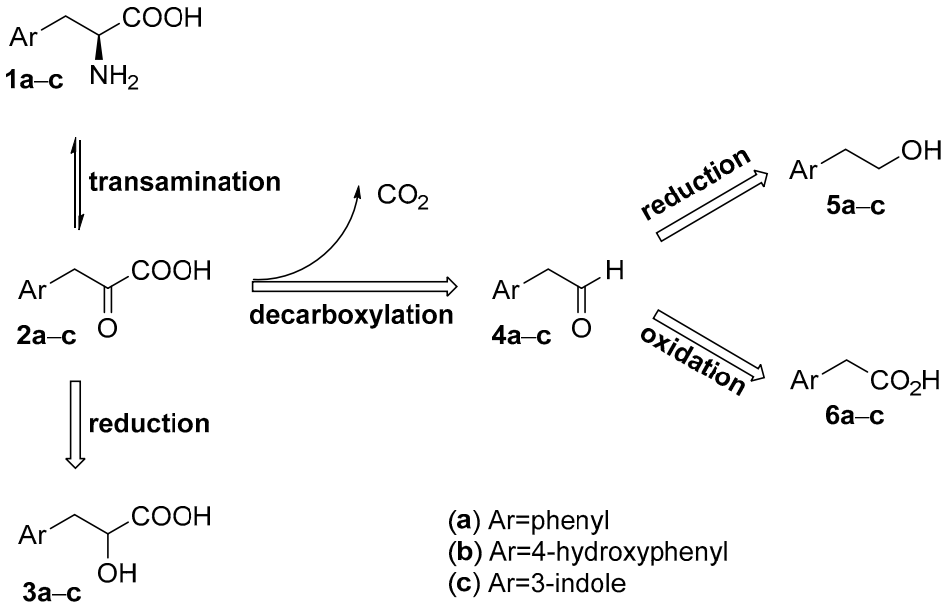

Figure 1. The biotransformation of the amino acids (L)-phenylalanine **1a**, (L)-tyrosine **1b** and (L)-tryptophan **1c** through Ehrlich pathway.

As described for alcohol **5a**, also PAA is an important flavour (pp. 1361–1362, 1656–1657, [14]) that is used in foods and beverages for its sweet, honey-like odour. Therefore, a number of biocatalytic syntheses of both latter compounds and of their derivatives have been developed so far, including those based on metabolic engineering [16–22].

All the processes described above fulfil the requirements of the European [23] and USA [24] legislations concerning natural flavour production. Indeed, both regulations establish that the biotransformation of a natural precursor is a 'natural method' of synthesis [25] and the flavours obtained by these means possess the 'natural' status. Since the flavours possessing the 'natural' status are usually hundreds of times as expensive as their synthetic counterparts, any new procedure that provides these compounds in their high-value form can be very profitable. This consideration is certainly pertinent for PAA, whose commercial value range from less than one hundred euros for the synthetic compound to more than a thousand euros for the flavour of natural origin.

We have already developed analytic methods of origin authentication of the most relevant natural phenylpropanoids, including PAA, by means of their stable isotope characterisation [26–30]. In addition, we have been recently involved in a research program aimed to develop new biocatalytic syntheses of high-value natural flavours [31–34].

Taking advantage of our acquired experience, we decided to study the yeasts-mediated transformation of the most relevant proteogenic aromatic amino acids, namely phenylalanine, tyrosine and tryptophan. Our preliminary results indicated a remarkable diversity among yeast species and we first discovered that different yeast strains belonging to the genus *Yarrowia* are able to transform directly phenylalanine **1a** into PAA.

Hence, we exploited these findings and devised a new preparative process for the synthesis of natural PAA [35]. Afterward, we extended our research by studying the activity of *Yarrowia lipolytica* in the biodegradation of both racemic and (D)-phenylalanine, as well as in the transformation of tyrosine and tryptophan.

We reported here the results of these investigations, which point to the great potential of *Yarrowia* strains in aromatic amino acids transformation.

2. Results and Discussion

As mentioned before, at first we investigated the yeast-mediated transformation of (L)-phenylalanine into PE and PAA. Since the main application of these compounds is their use as flavours in foods and beverages, we selected yeast strains belonging to biosafety level 1, with a strong preference for those recognised with technologically beneficial use in foods [36]. Accordingly, we set up a preliminary screening experiment in which four different yeast strains are fermented in the universal medium for yeasts (YM) containing (L)-phenylalanine (5 g/L), in an aerated flask (Table 1).

Table 1. Biotransformation of (L)-phenylalanine with short contact time and low aeration.

Entry [1]	Yeast	PE/PAA Ratio	PAA (g/L) [6]	PE + PAA Yield (%) [7]
1	*Saccharomyces cerevisiae* [2]	98/2	0.011	15.3
2	*Saccharomyces boulardi* [2,3]	97/3	0.013	13.1
3	*Pichia pastoris* [2]	97/3	0.013	14.8
4	*Yarrowia lipolytica* (DSM 8218) [2]	21/79	0.470	16.1
5	*Yarrowia lipolytica* (DSM 8218) [4,5]	<1/99	1.675	40.0

[1] Reaction conditions: YM containing 5 g/L of (L)-phenylalanine, at 26 °C for 3 days, under shaking (110 rpm). [2] Low aeration conditions: 100 mL of medium in 250 mL Erlenmeyer flask sealed with a cellulose plug. [3] The probiotic strain *Saccharomyces boulardi* I-3799 was grown at 36 °C. [4] High aeration conditions: 100 mL of medium in 1 L Erlenmeyer flask sealed with a cellulose plug. [5] 5 Days of fermentation. [6] Concentration of PAA in the fermentation broth after 3 days, measured by GC-MS analysis, using 2-phenylpropanoic acid as internal standard. [7] Molar yield of PE + PAA formation. The concentrations of PE and PAA in the fermentation broth were measured by GC-MS analysis, using 2-phenyl-1-propanol and 2-phenylpropanoic acid, respectively, as internal standards.

The rationale behind the choice of the four strains lies in the fact that *Saccharomyces cerevisiae*, the main yeast present in baker's yeast, has been historically used for PE production [1,2] and thus can be regarded as a 'positive control' in screening experiments. *Saccharomyces boulardi* is genetically correlated to *S. cerevisiae*, but this probiotic microorganism [37] can grow at a higher temperature (36 °C), allowing to investigate the biotransformation of phenylalanine at a temperature rather unusual for yeasts.

Pichia pastoris [38] is used in the food industry, as a model organism for biological studies and as a system for the expression of cloned genes. Even more interesting is the case of *Yarrowia lipolytica*, which has been recognised as safe for human health [39] and has been included in the list of GRAS microorganisms. This yeast is classified as oleaginous [40] as it is able to accumulate intracellularly a considerable amount of lipids. It has found a number of applications in industry and some strains have been employed for the transformation of phenylalanine into PE [41,42].

Our biotransformation experiments highlighted that *S. cerevisiae*, *S. boulardi* and *P. pastoris* transformed (L)-phenylalanine in PE (entries 1–3) with high selectivity since the PE/PAA ratio was superior to 97/3 for all cases. On the contrary, *Y. lipolytica* produced mainly PAA (entry 4) as the measured PE/PAA ratio was 21/79. The latter data disagree with those reported in the literature [41,42] opening the question about the influence of the experimental conditions and of the strain variability on the yeast activity.

As the PAA formation is subject to an oxidation reaction, we set a fifth experiment (entry 5) in which the biotransformation was performed with higher aeration conditions and with longer contact time (5 days instead of 3). The results of the latter trial were remarkable, indicating the importance of the oxygen supply for *Y. lipolytica* activity. Almost only PAA was formed, whereas the overall yield increased from 16.1% (entry 4) to 40.0%.

Taking into account the importance of the latter results, we performed a new set of experiments that examined a larger selection of yeast strains, whose activities, in high aeration conditions, were compared with that of the *Y. lipolytica* strain used before (DSM 8218).

Besides the four microorganisms described in Table 1, we singled out other oleaginous yeasts, belonging either to Ascomycota or to Basidiomycota division. In this context, we utilised *Candida boidinii*, *Starmerella bombicola*, *Debaryomyces hansenii*, *Cryptococcus curvatus*, *Sporidiobolus johnsonii*, and *Phaffia rhodozyma*, which have been largely employed in industrial processes [40,43–46] for the preparation of a number of food additives including

flavours and dietary supplements. Finally, within non-oleaginous yeasts, we selected two important microorganisms largely used in food transformation, namely *Torulaspora delbrueckii* [47] and *Kluyveromyces marxianus* [48].

Accordingly, the biotransformation experiments were performed using a starting concentration of phenylalanine of 4.5 g/L, high aeration conditions, and a rather long contact time (7 days). The results are collected in Table 2 and allow some important comments. Firstly, we can observe that the new biotransformation conditions do not affect the PE/PAA ratio only for *Saccharomyces* strains (entries 1 and 2) where PE remain almost the exclusive product. Otherwise, there is high variability in PAA productivity for all other strains tested. *Debaryomyces hansenii* and *Torulaspora delbrueckii* (entries 7 and 8) transform phenylalanine mainly into PE. *Pichia pastoris* and *Kluyveromyces marxianus* (entries 3 and 9) produced more PE than PAA, *Candida boidinii* and *Phaffia rhodozyma* (entries 5 and 12) produced more PAA than PE, whereas *Yarrowia lipolytica* and *Starmerella bombicola* (entries 4 and 6) produce almost exclusively PAA.

Table 2. Biotransformation of (L)-phenylalanine with long contact time and high aeration.

Entry [1]	Yeast	PE/PAA Ratio	PAA (g/L) [4]	PE + PAA Yield (%) [5]
1	*Saccharomyces cerevisiae*	97/3	0.02	16.8
2	*Saccharomyces boulardii* [2]	96/4	<0.01	3.7
3	*Pichia pastoris*	52/48	0.41	24.4
4	*Yarrowia lipolytica* (DSM 8218)	<1/99	1.97	53.0
5	*Candida boidinii*	35/65	0.87	37.9
6	*Starmerella bombicola*	<1/99	0.05	1.3
7	*Debaryomyces hansenii*	88/12	0.01	3.0
8	*Torulaspora delbrueckii*	87/13	0.06	13.4
9	*Kluyveromyces marxianus*	61/39	0.61	45.1
10	*Cryptococcus curvatus*	[3]	[3]	-
11	*Sporidiobolus johnsonii*	[3]	[3]	-
12	*Phaffia rhodozyma*	39/61	0.02	0.9

[1] Reaction conditions: YM containing 4.5 g/L of (L)-phenylalanine, at 26 °C for 7 days, under shaking (140 rpm). High aeration conditions: 100 mL of medium in 1 L Erlenmeyer flask sealed with a cellulose plug. [2] The probiotic strain *Saccharomyces boulardi* I-3799 was grown at 36 °C. [3] PE and PAA are not present in the broth after 7 days of fermentation. [4] Concentration of PAA in the fermentation broth after 7 days, measured by GC-MS analysis, using 2-phenylpropanoic acid as internal standard. [5] Molar yield of PE + PAA formation. The concentrations of PE and PAA in the fermentation broth were measured by GC-MS analysis, using 2-phenyl-1-propanol and 2-phenylpropanoic acid, respectively, as internal standards.

Besides the PE/PAA ratio, we took into account also the overall productivity (PE + PAA yield). Indeed, all Basidiomycota strains (entries 10–12), *S. bombicola*, and *D. hansenii* (entries 6 and 7) show very low productivity (up to 3% overall yield) most likely due to the almost complete utilisation of phenylalanine by fungal metabolism. Furthermore, the Ascomycota *Saccharomyces* strains, *P. pastoris*, and *T. delbrueckii* (entries 1–3 and 8) show overall yields that range from low to modest (up to 25%) whereas *Y. lipolytica*, *C. boidinii*, and *K. marxianus* (entries 4, 5, and 9) give the best performance in term of PE + PAA production (up to 53% total yield).

Overall, *Y. lipolytica* (DSM 8218) proved to be the most suitable microorganism for the transformation of (L)-phenylalanine into PAA as it showed a very high chemical selectivity (PAA/PE > 99/1) and good biotransformation yield (53%).

After establishing the potential of *Y. lipolytica* for PAA synthesis, we investigated the tolerance of the same strain to higher substrate concentrations. Indeed, in order to reduce bioreactor volume, industrial syntheses based on fermentation processes should be performed with high substrate concentrations. Therefore, we ran five biotransformation experiments in which *Y. lipolytica* (DSM 8218) was fermented using high aeration conditions, a rather long contact time (7 days), and different concentrations of the substrate. The phenylalanine concentration ranged from 4.5 to 25 g/L (Table 3, entries 1–5).

Table 3. Synthesis of phenylacetic acid by biotransformation of (L)-phenylalanine, at different concentrations, using *Yarrowia lipolytica* (DSM 8218).

Entry [1]	(L)-Phenylalanine Starting Concentration (g/L)	PAA Yield (%) [2]
1	4.5	51.0
2	8.5	55.4
3	12.5	67.1
4	18.0	52.6
5	25.0	24.2

[1] Reaction conditions: YM containing (L)-phenylalanine, at 28 °C for 7 days, under shaking (140 rpm). High aeration conditions: 100 mL of medium in 1 L Erlenmeyer flask sealed with a cellulose plug. [2] Yields are determined after work-up by isolation of the formed PAA.

The experiment performed at the lower substrate concentration (entry 1, 4.5 g/L) correspond to that described in Table 2, with entry 4. The measured yields are almost identical, indicating a good reproducibility of the experimental procedure.

According to the data collected in the table, we observed that the increase of the substrate concentration at first corresponded to an increase of the PAA isolated yields (entries 1–3), up to 67.1%. Then, higher phenylalanine concentrations initially gave a slightly lower yield (entry 4) and finally produced a drastic reduction of the same (entry 5). Overall, we can conclude that *Y. lipolytica* gives the best PAA yields with a substrate concentration of around 12.5 g/L. Although fermentation is possible even at an initial concentration of 25 g/L, we observed a considerable transformation inhibition, most likely due to the toxic effect of the substrate itself.

All the above-described experiments have been performed using the same *Y. lipolytica* strain (DSM 8218). Since the latter microbial strain possesses a specific ability to oxidise aromatic hydrocarbons [49], we could not exclude that the aptitude of *Y. lipolytica* to produce PAA was dependent on strain origin and on its genetic variability. Thus, we decided to investigate whether other *Yarrowia* strains or species are able to perform the same biotransformation. It is worth noting that since its first description [50], the genus *Yarrowia* was monotypic, accommodating the single species *Y. lipolytica*. Only recently, the new molecular techniques of microbial typing allowed the introduction of numerous novel species [51–55].

In order to study their potential use for PAA synthesis, we thus selected different species belonging to *Yarrowia* genus and one further strain of *Y. lipolytica*. More specifically, we singled out a number of strains that are placed in different positions of the last described *Yarrowia* clade phylogenetic tree [55] or that were isolated from very different natural environments. All the selected strains were employed in biotransformation experiments with high aeration conditions, a rather long contact time (7 days), and a phenylalanine concentration of 12.5 g/L (Table 4). Accordingly, we ran seven flask fermentation trials (entries 1–7).

The two *Y. lipolytica* strains (entries 1 and 2), the first one isolated from a fuel storage tank (DSM 8218) and the second one isolated from marzipan (DSM 70562), gave almost identical results regardless of the different isolation substrates, affording PAA in 67 and 69% molar yield, respectively. Similarly, *Y. deformans* and *Y. bubula* (entries 3 and 5), which hold well-separated positions in *Yarrowia* phylogenetic tree, afforded PAA in 64 and 66% molar yield, respectively. Differently, *Y. yakushimensis*, *Y. phangngaensis*, and *Y. brassicae* species (entries 4, 6, and 7), gave disappointing results affording PAA in 24, 27, and 32% yield, respectively. Seen together, these results indicate that neither the strain origin nor the genetic similarity between species can be used as the sole parameter to identify the best PAA producers in *Yarrowia* clade.

Overall, we selected *Y. lipolytica*, *Y. deformans*, and *Y. bubula* as the best *Yarrowia* species for the production of natural PAA. Accordingly, we used the corresponding four strains (entries 8–11) for the preparative scale synthesis of PAA. To this end, we performed the biotransformations in the bioreactor, using the same experimental conditions employed

for flask fermentations. For all strains, we observed a definite increase in PAA yield, most likely due to the better control of the aeration and of the pH in the bioreactor.

Table 4. Synthesis of PAA acid by biotransformation of (L)-phenylalanine using different *Yarrowia* strains.

Entry [1]	*Yarrowia* Strain	Fermentation Conditions	PAA Yield (%) [4]
1	*Yarrowia lipolytica* (DSM 8218)	Flask [2]	67
2	*Yarrowia lipolytica* (DSM 70562)	Flask [2]	69
3	*Yarrowia deformans* (DSM 70561)	Flask [2]	64
4	*Yarrowia yakushimensis* (CBS 10252)	Flask [2]	24
5	*Yarrowia bubula* (CBS 12934)	Flask [2]	66
6	*Yarrowia phangngaensis* (CBS 10407)	Flask [2]	27
7	*Yarrowia brassicae* (CBS 15225)	Flask [2]	32
8	*Yarrowia lipolytica* (DSM 8218)	Bioreactor [3]	71
9	*Yarrowia lipolytica* (DSM 70562)	Bioreactor [3]	75
10	*Yarrowia deformans* (DSM 70561)	Bioreactor [3]	72
11	*Yarrowia bubula* (CBS 12934)	Bioreactor [3]	68

[1] Reaction conditions: YM containing 12.5 g/L of (L)-phenylalanine, at 28 °C for 7 days. [2] Flask fermentation: 100 mL of medium in 1 L Erlenmeyer flask sealed with a cellulose plug, under shaking (140 rpm). [3] Bioreactor fermentation: 5 L fermenter loaded with 2 L of medium. Airflow, stirring speed, and pH were set to 1 L/min, 250 rpm, and 6.5, respectively. [4] Yields are determined after work-up by isolation of the formed PAA.

Then, all the findings above described, allowed us to patent [35] a new preparative process for the synthesis of natural PAA starting from natural (L)-phenylalanine. Our approach compares favourably with the previously reported whole-cell procedures based on the direct transformation of phenylalanine into PAA [3,4]. Indeed, the latter approaches claimed inferior yields and make use of microorganisms not suitable for food flavour production, as those belonging to BSL 2 [3]. On the contrary, our process takes advantage of *Yarrowia* strains possessing the GRAS status and producing natural PAA in isolated yields ranging from 70 to 75%. Moreover, our method is user-friendly, the isolation of the product does not require difficult separation procedures and its commercial viability is secured by the high difference in value between the starting material, namely natural (L)-phenylalanine (less than 200 euro/kg), and the produced natural PAA (more than 1000 euro/kg).

A further extension of our research was the study of the transformation of the amino acid phenylalanine in relation to its enantiomeric composition. Since the first step in the Ehrlich pathway is the transamination reaction, which is catalysed by different transaminases, the yeast could not able to transform the unnatural (D)-enantiomer. Indeed, the latter enzymes usually catalyse the preferential transformation of (L)-phenylalanine with high enantioselectivity.

Thus, we arranged a new set of biotransformation trials that employed the same experimental conditions and different substrate compositions. Accordingly, either (L)-, (DL)- and (D)-enantiomers of phenylalanine, in the universal medium for yeasts (YM) and with a starting concentration of 10 g/L, were fermented using *Y. lipolytica* (Table 5, entry 1–3). As previously described, (L)-phenylalanine was converted into PAA (entry 1) in good yield (53.4%) whereas the transformation of the racemic amino acid (entry 2) was more difficult, affording PAA in only 25.5% yield. These data could be explained assuming that *Y. lipolytica* is either unable to transform the (D)-isomer or the transformation rate of the latter enantiomer is much inferior to that of (L)-isomer. To investigate better this point, we examined the biotransformation experiments in which only (D)-phenylalanine was present in the fermentation broth. We observed that also in this case (entry 3) PAA was formed, even if in a lower amount (12.1%). Since yeast could able to uptake (L)-phenylalanine from the proteins present in the medium, we repeated the last experiment using a protein-free medium (PF-YM), which contain neither proteins nor (L)-phenylalanine. As a result, PAA was yet produced although the isolated yield was further reduced to 5.5% (entry 4).

Table 5. Synthesis of phenylacetic acid by biotransformation of (L)-, (DL)-, and (D)-phenylalanine, using *Yarrowia lipolytica* (DSM 8218).

Entry [1]	Phenylalanine Enantiomer	Fermentation Medium	PAA Yield (%) [2]
1	(L)	YM	53.4
2	(DL)	YM	25.5
3	(D)	YM	12.1
4	(D)	PF-YM	5.5

[1] Reaction conditions: YM or PF-YM containing 10 g/L of phenylalanine, at 28 °C for 7 days, under shaking (140 rpm). High aeration conditions: 100 mL of medium in 1 L Erlenmeyer flask sealed with a cellulose plug. [2] Yields are determined after work-up by isolation of the formed PAA.

Overall, these results demonstrated that *Y. lipolytica* preferentially converted (L)-phenylalanine into PAA whereas the transformation of the (D)-enantiomer was very slow. This behaviour could be explained either by invoking the production of different transaminases during *Yarrowia* fermentation or by considering the possibility of a slow process of amino acid racemisation within microbial catabolism of the same.

As a final point, we investigated the potential of *Y. lipolytica* in the transformation of two further relevant proteogenic aromatic amino acids, namely tyrosine and tryptophan. To this end, we devised three experiments in which the same *Y. lipolytica* strain was fermented in presence of either (L)-phenylalanine, (L)-tyrosine, or (L)-tryptophan, employing the same experimental conditions. In order to use the same amino acid concentration for all trials, we set a value of 5 g/L for each trial, which was the highest possible concentration in YM for the less soluble amino acid tyrosine.

Accordingly, the results of the biotransformation trials showed very interesting results (Table 6).

Table 6. Biotransformation of (L)-phenylalanine, (L)-tyrosine and (L)-tryptophan using *Yarrowia lipolytica* (DSM 8218).

Entry [1]	Amino Acid	Compounds 2–6 [2]	Other [2]
1	(L)-Phenyl alanine	**6a** (58.3%)	-
2	(L)-Tyrosine	**6b** (47.7%)	-
3	(L)-Tryptophan	**3c** (16.0%); **6c** (1.3%)	**Anthranilic acid** (33.6%)

[1] Biotransformation conditions: 200 mL of YM medium containing 5.0 g/L of the suitable amino acid, at 26 °C for 7 days, under shaking (140 rpm), in 1 L Erlenmeyer flask sealed with a cellulose plug. [2] The compounds were identified by GC-MS analysis. Molar yields (%) are determined after work-up by chromatographic isolation of the products.

First, we observed that (L)-phenylalanine and (L)-tyrosine were transformed in the same way affording exclusively the corresponding arylacetic acid, namely PAA **6a** and 4-hydroxyphenyl acetic acid **6b**, in 58 and 48% yield, respectively (entries 1 and 2). It is worth noting that 4-hydroxyphenyl acetic acid is a relevant bioactive compound possessing antioxidant [11] and anti-inflammatory [12] properties. Despite this fact, its synthesis from tyrosine by whole cell process has not been reported yet. Only very recently [56], research on the transformation of lignin-related phenylpropanoids has described the biotransformation of *p*-cumaric acid into **6b** using engineered *E. coli*.

As a final point, we observed that *Y. lipolytica* transformed (L)-tryptophan in a completely different way (entry 3). Indolacetic acid **6c** was the minor metabolite, generated in a very minute amount (1.3% yield). Even for this trial, neither 2-indole-ethanol **5c** nor indole-3-pyruvic acid **2c** was formed. Differently, indole-3-lactic acid **3c** was produced in significant amounts (16% yield) whereas anthranilic acid (2-aminobenzoic acid) turned out to be the major product synthesised from (L)-tryptophan **1c** by *Y. lipolytica*. The latter compound is most likely produced through a different pathway that involves the oxidation of the indole ring, not necessarily related to the Ehrlich pathway. It should be pointed out that both methyl and ethyl esters of the aforementioned acid are relevant flavours

([14], pp. 569, 1213 –1214). Therefore, the *Y. lipolytica* mediated transformation of natural tryptophan could represent new access to the latter flavours in natural form.

In conclusion, the present study demonstrates that different yeast strains belonging to *Yarrowia* genus metabolise the aromatic amino acids (L)-phenylalanine and (L)-tyrosine with the almost exclusive formation of phenylacetic acid **6a** and 4-hydroxyphenylacetic acid **6b**, respectively, in good yield.

Except for minor differences in terms of yield, both biotransformation processes hold preparative significance, as they are easily scalable from flask trial to bioreactor production and the produced arylacetic acids can be isolated from the fermentation broth, in almost pure form, by simple extraction with a suitable solvent. By these means, employing as substrates (L)-phenylalanine of natural origin, the obtained acid can be labelled as natural allowing the production of the high-value flavour phenylacetic acid. On the contrary, the transformation of (L)-tryptophan with *Y. lipolytica* gave indoleacetic acid **6c** in a trace amount. For this biotransformation, anthranilic acid was the main product, alongside a minor amount of indole-3-lactic acid **3c**. Since the esters of anthranilic acid are natural flavours, the yeast-mediated transformation of (L)-tryptophan deserves further consideration and will be the subject of our next studies.

3. Materials and Methods

3.1. Materials and General Methods

All air and moisture-sensitive reactions were carried out using dry solvents and under a static atmosphere of nitrogen. All solvents and reagents were of commercial quality and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2-Phenylpropanoic acid was prepared by carbonation of (1-phenylethyl)-magnesium bromide, in turn, obtained by reaction of (1-bromoethyl)benzene with magnesium turning.

The methyl esters of 2-phenylpropanoic acid **8**, phenylacetic acid **12**, 4-hydroxyphenyl acetic acid **15**, 3-indole acetic acid **18**, phenyl pyruvic acid **10**, 4-hydroxyphenyl pyruvic acid **13**, indole-3-pyruvic acid **16**, 3-phenyllactic acid **11**, 3-(4-hydroxyphenyl)lactic acid **14**, indole-3-lactic acid **17**, and anthranilic acids **9** (Figure 2) were prepared by treatment at 0 °C of the corresponding acid with an excess of an ethereal solution of freshly-prepared diazomethane.

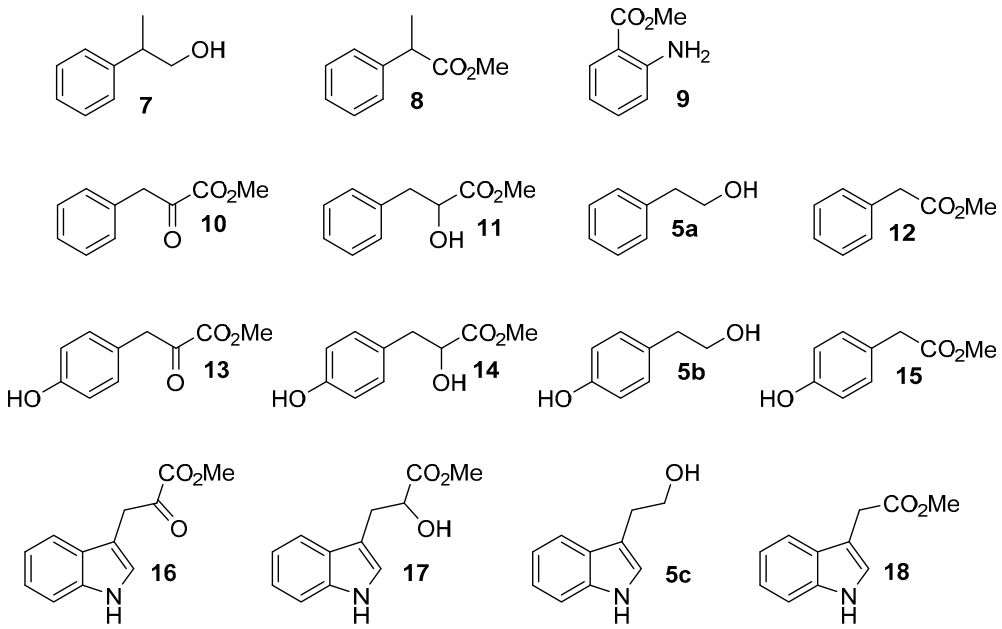

Figure 2. Reference standards used for GC-MS analysis of the biotransformation experiments.

3.2. Microorganisms and Growth Media

Saccharomyces cerevisiae (Type II YSC2, Lot BCBR1308) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Saccharomyces boulardii (probiotic strain SB80®, I-3799) was purchased from AR Fitofarma s.r.l (Assago, Italy).

Pichia pastoris (*Komagataella pastoris,* DSM 70382), *Yarrowia lipolytica* (DSM 8218), *Yarrowia lipolytica* (DSM 70562), *Yarrowia deformans* (DSM 70561), *Torulaspora delbrueckii* (DSM 70483), *Debaryomyces hansenii* (DSM 70590), *Candida boidinii* (DSM 70026), *Starmerella bombicola* (DSM 27465), *Kluyveromyces marxianus* (DSM 70073), *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*, DMS 5626), *Cryptococcus curvatus* (DSM 70022), and *Sporidiobolus johnsonii* (DSM 70580) were purchased from DSMZ GmbH collection (Braunschweig, Germany).

Yarrowia yakushimensis (CBS 10252), *Yarrowia bubula* (CBS 12934), *Yarrowia phangngaensis* (CBS 10407), and *Yarrowia brassicae* (CBS 15225) were purchased from the CBS-KNAW collection (Utrecht, The Netherlands).

The biotransformation experiments were performed using two different media, namely a universal medium for yeasts (YM) and a protein-free medium for yeasts (PF-YM), depending on the microorganism used.

YM composition: yeast extract (3 g/L), malt extract (3 g/L), peptone from soybeans (5 g/L), glucose (10 g/L), trace element solution (10 mL).

PF-YM composition: glucose (10 g/L), soluble starch (3 g/L), maltose (2 g/L), yeast nitrogen base (0.7 g/L), trace element solution (10 mL).

Trace elements solution: $FeCl_3$ (50 mM), $CaCl_2$ (20 mM), $MnCl_2$ (10 mM), $ZnSO_4$ (10 mM), $CoCl_2$ (2 mM), $CuCl_2$ (2 mM), $NiCl_2$ (2 mM), Na_2MoO_4 (2 mM), Na_2SeO_3 (2 mM), H_3BO_3 (2 mM).

*3.3. Biotransformation Experiments and Preparative Synthesis of Phenylacetic Acid **6a** and 4-Hydroxy-Phenylacetic Acid **6b***

All the biotransformation experiments were carried out in triplicate and the results presented in the tables are the media of three experimental data. The experiments were performed either in flasks or in a 5 L fermenter (Biostat A BB-8822000, Sartorius-Stedim, Göttingen, Germany).

Phenylalanine, tyrosine, and tryptophan were added to the suitable medium before the sterilisation cycle (121 °C, 20 min).

The inoculum of the yeast strains was prepared according to the following procedure:

A small amount of the suitable yeast strain was picked up from a petri dish, suspended in 1 mL of sterile saline, and then inoculated in a 100 mL conical pyrex flask containing 40 mL of YM. The flask was shaken for 3 days at 26 °C and 140 rpm. After this period, 1 mL of this active culture was used to inoculate 40 mL of the fresh medium containing the suitable amino acid. For preparative biotransformations, the whole active culture described above was centrifuged 5 min, (4 °C, 3220× *g*). Cells were collected, removed from the medium, suspended in 5 mL of sterile saline, and inoculated in 1 L of YM containing (L)-phenylalanine or (L)-tyrosine.

3.3.1. General Procedure for Biotransformation Experiments Using Aerobic Flasks

The aerobic flasks were prepared by loading 100 mL of the suitable medium (YM or PF-YM) containing the given amino acid in 300 mL (for experiments with reduced aeration) or 1 L conical Erlenmeyer flasks. The flasks were sealed with a cellulose plug and sterilised (121 °C, 15 min.). Each flask was inoculated with the active culture of the suitable yeast, and then it was shacked and incubated at the temperature and for the time indicated in the tables. Hence, the reaction mixtures were analysed according to the procedures described below (3.4.1 or 3.4.2) or were worked up in order to isolate the biotransformation products. Accordingly, the fermentation broths were acidified at pH 4 by the addition of diluted HCl and then filtered on celite. The aqueous phases are then extracted three times with ethyl acetate and the combined organic layers were washed with brine, dried (Na_2SO_4)

and the solvent was removed under reduced pressure. The residue was then purified by chromatography.

3.3.2. Bioreactor Based Preparative Synthesis of Phenylacetic Acid **6a** and 4-Hydroxy-Phenylacetic Acid **6b**

A 5 L fermenter was loaded with YM (2 L) containing natural (L)-phenylalanine (12.5 g/L). The bioreactor was sterilised at 121 °C for 15 min, then the temperature, the stirring speed, and the pH were set to 28 °C, 250 rpm, and 6.5, respectively. The pH was controlled by the dropwise addition of sterilised aqueous solutions (10% *w*/*w* in water) of either acetic acid or ammonia. Hence, we added a suitable inoculum of *Yarrowia lipolytica* (DSM 8218), and the fermentation continued for 7 days whilst aeration was secured by a continuous airflow (1 L/min). The biotransformation was then stopped by the addition of concentrated HCl until a pH of 4.0–4.5 is reached. The fermentation broth is filtered through celite and extracted with ethyl acetate (4 × 200 mL). The combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by distillation to give pure (97% by GC-MS analysis) phenylacetic acid **6a** (15.0 g, 71% yield)

The same procedure was performed using *Yarrowia lipolytica* (DSM 70562) and *Yarrowia deformans* (DSM 70561) strains, to afford phenylacetic acid **6a** in 75% and 72% yield, respectively.

The same procedure described above was used for the biotransformation of (L)-tyrosine. In this experiment, we fermented *Yarrowia lipolytica* (DSM 8218), in presence of a starting amino acid concentration of 5 g/L. The residue obtained after work-up and extraction was purified by chromatography to afford pure 4-hydroxy-phenylacetic acid **6b** (4.95 g, 59% yield).

M.p. = 151–152 °C

^{1}H NMR (400 MHz, $CDCl_3$): δ = 12.13 (br s, 1H), 9.24 (br s, 1H), 7.04 (d, *J* = 8.4 Hz, 2H), 6.70 (d, *J* = 8.4 Hz, 2H), 3.43 (s, 2H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 173.0 (C), 156.0 (C), 130.2 (CH), 125.1 (C), 114.9 (CH), 39.8 (CH_2).

MS (ESI): 175.1 ($M + Na^+$); 150.9 ($M-H^+$).

Copies of the ^{1}H and ^{13}C-NMR spectra and of the ESI-MS spectra of 4-hydroxyphenylacetic acid **6b** are reported as Supplementary Materials.

3.4. Analytical Methods and Characterisation of the Products Deriving from the Biotransformation Experiments

Nuclear Magnetic Resonance spectroscopy (NMR): ^{1}H- and ^{13}C-NMR Spectra and DEPT experiments: $CDCl_3$ solutions at room temperature (rt) using a Bruker-AC-400 spectrometer (Billerica, MA, USA) at 400, 100, and 100 MHz, respectively; ^{13}C spectra are proton decoupled; chemical shifts in ppm relative to internal $SiMe_4$ (=0 ppm).

TLC: Merck silica gel 60 F_{254} plates (Merck Millipore, Milan, Italy).

Column chromatography: silica gel.

Melting points were measured on a Reichert apparatus (Reichert, Vienna, Austria), equipped with a Reichert microscope, and are uncorrected.

Mass spectra were recorded on a Bruker ESQUIRE 3000 PLUS spectrometer (ESI detector) (Billerica, MA, USA) or by GC-MS analyses.

GC-MS analyses: A HP-6890 gas chromatograph equipped with a 5973 mass detector and using an *HP-5MS* column (30 m × 0.25 mm, 0.25 μm film thickness; Hewlett Packard, Palo Alto, CA, USA) was used with the following temp. program: 60° (1 min)–6°/min–150° (1 min)–12°/min–280° (5 min); carrier gas: He; constant flow 1 mL/min; split ratio, 1/30; t_R given in min:

t_R(**7**) 10.90, t_R(**8**) 11.73, t_R(**9**) 15.63, t_R(**10**) 15.41, t_R(**11**) 15.90, t_R(**5a**) 9.58, t_R(**12**) 11.13, t_R(**13**) 21.55, t_R(**14**) 21.79, t_R(**5b**) 17.69, t_R(**15**) 18.90, t_R(**16**) 25.70, t_R(**17**) 25.76, t_R(**5c**) 23.10, t_R(**18**) 23.77.

Copies of the MS spectra of phenylacetic acid methyl ester **12**, phenylethanol **5a**, 4-hydroxyphenylacetic acid methyl ester **15**, methyl anthranilate **9**, indole-3-lactic acid methyl ester **17** and 3-indole acetic acid methyl ester **18** are reported as Supplementary Materials.

The biotransformation reactions were analysed using two different procedures depending on whether the internal standard was used or not.

3.4.1. Extraction/Analysis Procedure (without Internal Standard)

The biotransformation broth was treated at 0 °C with concentrated HCl aq. (37% *w*/*v*) in order to bring the pH between 3 and 4. Ethyl acetate (about one-fourth of the volume of the broth) was added and the mixture was filtered on a celite pad. The celite-biomass cake was washed with acetate, the phases were separated, and the aqueous phase was extracted with further solvent. The combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure. The residue was treated with an excess of an ethereal solution of freshly-prepared diazomethane and then submitted to GC-MS analysis.

3.4.2. Quantitative Analysis Procedure (with Internal Standard)

2-Phenyl-1-propanol (500 µL of a 50 g/L solution in ethanol) and 2-phenylpropanoic acid (500 µL of an 80 g/L solution in ethanol) were added to a 39 mL sample of the biotransformation broth. The mixture was stirred at rt for 10 min, then was acidified (final pH 3–4) by dropwise addition of concentrated HCl aq. (37% *w*/*v*). Ethyl acetate (10 mL) was added, and the mixture was vigorously stirred for half an hour. The mixture was centrifuged for 5 min, (4 °C, 3220× *g*) and the organic phase was collected, dried (Na_2SO_4), and concentrated in vacuo. The residue was treated with an excess of an ethereal solution of freshly-prepared diazomethane and then submitted to GC-MS analysis. The internal standards 2-phenyl-1-propanol **7** and 2-phenylpropanoic acid methyl ester **8** were present in the fermentation broth at a concentration of 0.625 g/L and 1 g/L, respectively, and their peaks were used to determine the concentration of 2-phenylethanol **5a** and of phenylacetic acid **6a** in the same biotransformation mixture.

4. Patents

The work reported in this manuscript is partially covered by the following international patent: 'Process for preparing phenylacetic acid', International Publication Number: WO 2021/205299 A1; Application Number: PCT/IB2021/052744; Priority Data: 6 April 2020; International Publication Date: 14 October 2021; Applicant: Consiglio Nazionale delle Ricerche (CNR), Piazzale Aldo Moro, 7, 00195 Roma; Inventors: Castagna Antonio, Serra Stefano, Valentino Mattia.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/catal12121638/s1, Figure S1: ^{1}H NMR of 4-hydroxyphenylacetic acid **6b**; Figure S2: ^{13}C NMR of 4-hydroxyphenylacetic acid **6b**; Figure S3: ESI-MS of 4-hydroxyphenylacetic acid **6b**; Figure S4: MS spectra of phenylacetic acid methyl ester **12**; Figure S5: MS spectra of phenylethanol **5a**; Figure S6: MS spectra of 4-hydroxyphenylacetic acid methyl ester **15**; Figure S7: MS spectra of methyl anthranilate **9**; Figure S8: MS spectra of indole-3-lactic acid methyl ester **17**; Figure S9: MS spectra of 3-indole acetic acid methyl ester **18**.

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