Improved Production of Kynurenic Acid by Yarrowia lipolytica in Media Containing Different Honeys

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Abstract: Y. lipolytica remains a nonpathogenic, unconventional yeast, which can be applied for the production of bioactive compounds. Our previous study confirmed the ability of yeast Yarrowia lipolytica to produce kynurenic acid (KYNA). Here, we investigated the effectiveness of KYNA production in cultures cultivated in medium containing honey of various origin, used as a source of carbon and energy. It was evidenced that the highest content of KYNA in culture broth (68 mg/L) and yeast biomass (542 mg/kg) was obtained when chestnut honey was used. The content of lipids and amino acids composition in yeast biomass producing KYNA was also determined. It was found that the composition of both amino acids and lipids in yeast biomass depended on the honey type used as a component of the medium. This finding revealed that supplementation of medium broth with honey may significantly affect the nutritional value of yeast biomass. The practical applicability of this finding requires further study.

Keywords: kynurenic acid; honey; yeast; Yarrowia lipolytica

1. Introduction

Nowadays, a rising interest in finding new sources of bioactive compounds can be observed. Microbial sources of bioactive substances (as yeast) can be considered as alternative to traditional sources and they can be used for commercial purposes. Yarrowia lipolytica is an oleaginous yeast, which is able to accumulate high amounts of lipids, i.e., even 50% of cell dry weight [1]. However, Y. lipolytica is considered not only an interesting platform for lipid production, but also for the production of biomass [2], organic acids as citric acid [3,4] or sweeteners (e.g., erythritol) [5]. Recently, we discovered that Y. lipolytica yeast produces a tryptophan metabolite, kynurenic acid (KYNA) [6]. This compound is synthesized in the reaction of kynurenine transamination, which is catalyzed by kynurenine aminotransferases (KATs). KYNA exhibits pro-healthy properties, due to its antioxidant, anti-inflammatory and anticonvulsant action [7–9]. It was also reported that KYNA can have protective properties on the human brain [9] and an inhibitory effect on the proliferation of colon cancer cells (i.e., adenocarcinoma HT-29 cells) [8]. Despite its valuable properties, the main limitation of KYNA application is its low content in natural sources and low solubility in water and aqueous solutions [10]. Our former study showed that KYNA synthesized by yeast Y. lipolytica strain S12 is present in biomass and culture broth [6]. It should be pointed out that Y. lipolytica was noted as Generally Recognized
As Safe (GRAS) by the US Food and Drug Administration [11,12], and the biomass of Y. lipolytica is recognized as a novel food according to EU regulation 2015/2283. Thus, yeast biomass containing KYNA may serve as a promising, novel diet supplement. Based on our previous research it was exhibited that the most effective production of KYNA was in the medium containing fructose and tryptophan (the precursor for KYNA synthesis) [6]. Therefore, in the present research, the production of KYNA and accumulation of lipids and content of amino acids in yeast biomass was investigated when honey, a rich nutritional source of KYNA [13], was used as a source of carbon and energy.

2. Materials and Methods

2.1. Microorganisms

The Y. lipolytica yeast (strain S12) applied in the present research was derived from the Yeast Culture Collection of the Department of Biotechnology and Food Microbiology, Wroclaw University of Environmental and Life Sciences, Poland.

2.2. Conditions of Cultures

The used medium for inoculation cultures was composed of 10 g/L glucose, 3 g/L malt extract, 3 g/L yeast extract and 5 g/L peptone. The cultures were grown in a 300 mL flask at 29 °C for 72 h on a rotary shaker.

The production medium for bioreactor cultures contained: 40 g/L fructose or alternatively 50 g/L honey and 9 g/L (NH₄)₂SO₄, 0.3 g/L MgSO₄ × 7 H₂O, 0.25 g/L KH₂PO₄ and 1 g/L yeast extract. One of the culture types was supplemented with 200 mg/L of tryptophan. The pH was adjusted to 5.3 with 10% HCl solution (v/v). All used media were autoclaved at 121 °C for 20 min. Bioreactor cultures were conducted in a 5-L stirred-tank bioreactor (BIOSTAT B-PLUS, Sartorius, Goettingen, Germany) at 29 °C, stirred at 800 rpm and under aeration of 0.6 vvm. The volume of 200 mL of culture was used as inoculum for bioreactor cultures, which were performed in two replicates.

2.3. Determination of Dried Yeast Biomass

Five milliliters of culture broth was centrifuged (6000×g), washed with distilled water, filtered through 0.45 μm pore-size membranes and dried at 105 °C. Finally, the yeast biomass was expressed as g CDW/L (grams of cell dry weight per liter).

2.3.1. KYNA Detection via HPLC Method

KYNA was analyzed both in dried yeast biomass and culture broth in all conducted bioreactor cultures. The method was exactly the same as described in Wróbel-Kwiatkowska et al., 2020 [6].

2.3.2. Analysis of Protein Content and Amino Acids Profile in Yeast Biomass

Protein content in dry yeast biomass was determined via the standard method of the Association of Official Analytical Chemists International [14]. Total nitrogen was determined by the Kjeldahl method using a Büchi distillation unit K-355 (Athens, Greece). A nitrogen to protein conversion factor of 6.25 was used to calculate total protein. The amino acid composition was determined by ion exchange chromatography after hydrolysis. Ninhydrin was used for amino acid post-column derivatization [15,16]. An AAA-400 Automatic Amino Acids analyzer (INGOS Ltd., Prague, Czech Republic) provided with a double wavelength photometric detector and an Ostion LG ANB packed column 350 × 3.7 mm (INGOS) was used. The external standard method was used for quantifying amino acids. All the experiments were performed in duplicate.

2.3.3. Analysis of Fatty Acids in Yeast Biomass

Fatty acids were detected via the gas chromatography–mass spectrometry (GC–MS) method. Thus, the lyophilized yeast biomass was transesterified to fatty acid methyl esters (FAMEs) using the
protocol described in Browse et al., 1986 [17]. After derivatization using 2.5% sulfuric acid in methanol (containing 50 µg/mL of C17:0 as an internal standard), FAMEs were analyzed by GC–MS (Shimadzu, Kyoto, Japan) using Zebron ZB-FAME capillary column (30 m × 0.25 mm × 0.20 µm). Splitless injection mode was used for injecting the sample (1 µL at 250 °C) using helium (1 mL/min). Fatty acids were identified by comparing the retention time with reference compounds (Supelco 37 Component FAME mix, Sigma-Aldrich, St. Louis, MO, USA). All the experiments were performed in triplicate.

2.3.4. Statistical Analysis

Data are presented as means ± standard deviation (SD). Statistical analysis was performed with one-way ANOVA with the Tukey post-test (GraphPadInStat). p < 0.05 was considered significant.

3. Results and Discussion

3.1. KYNA Production in Media Containing Different Honeys

First, we investigated the synthesis of KYNA in media containing different types of honey: lime, acacia, honeydew, rape, chestnut and polyfloral honey. The quantitative analysis of KYNA via the HPLC method was carried out in culture broth and yeast biomass seven days after inoculation. Results are presented in Figure 1. The highest amounts of KYNA reaching 67.67 mg/L in culture broth and 541.8 mg/kg CDW were detected in the cultures performed in media containing chestnut honey. However, due to the high content of KYNA in chestnut honey, the de novo production of KYNA by yeast in the presence of this honey was 42.1 mg/L and accounted for 62% of the total content of KYNA in the medium (Figure 1A). In all other cultures performed with the honeys tested, de novo production of KYNA by yeast accounted for over 99% of the total amount of this compound in the medium. The lowest production of KYNA was noticed in the medium containing lime honey and the lowest content of KYNA in biomass was detected in yeast cultured on rape honey (Figure 1B).

Figure 1. Kynurenric acid (KYNA) synthesis in media containing different types of honey, applied as a carbon and energy source. KYNA content was determined in culture broth (A) and biomass of Y. lipolytica S12 (B), cultured in a bioreactor as described in Materials and Methods.

In our previous study [6], it was found that cultivation of Yarrowia lipolytica S12 under identical laboratory conditions in medium containing an optimal concentration of fructose (40 g/L) resulted in KYNA content reaching 7.9 mg/L and 39.5 mg/kg CDW in medium and biomass, respectively. Thus, in the present study, production of KYNA in cultures cultivated on chestnut honey was over 5 times higher in comparison to the medium containing fructose. Surprisingly, the content of KYNA in biomass was over 13 times higher in yeast incubated in the presence of chestnut honey in comparison to the fructose-containing medium.
The content of KYNA in medium from cultures performed on the remaining honeys is comparable to that obtained on fructose [6]. However, the content of KYNA in yeast biomass cultivated on honeys was approximately twice as high as on fructose [6]. Rape honey was an exception (Figure 1B).

3.2. Analysis of Sugar in Medium Containing Chestnut Honey

Since sugar is a main source of carbon and energy for yeast, we measured the content of fructose and glucose in broth throughout the entire incubation. Results are presented in Table 1.

Table 1. Content of fructose and glucose in the medium containing chestnut honey (50 g/L).

| Time [h] | Fructose [g/L] | Glucose [g/L] |
|----------|----------------|---------------|
| 0        | 13.96          | 7.85          |
| 24       | 4.21           | n.d.          |
| 48       | n.d.           | n.d.          |
| 72       | n.d.           | n.d.          |
| 96       | n.d.           | n.d.          |
| 120      | n.d.           | n.d.          |
| 168      | n.d.           | n.d.          |

Note: n.d.—not determined (below the detection limit).

It is evident that in our current study, the sum of both monosaccharides (21.8 g/L) is almost 2 times lower than the optimal concentration of fructose in the previous study (40 g/L). Moreover, the decay of monosaccharides from the culture medium (Table 1) was faster than from the medium with fructose (40 g/L) [6]. At this point, it should be stressed that the lower content of fructose (30 g/L) resulted in an almost 2 times lower content of KYNA in medium and an almost 8 times lower content of this compound in biomass [6]. Thus, taking into account the content of fructose and glucose, the production of KYNA in the medium with chestnut honey is much more efficient in comparison to the medium containing a comparable amount of fructose.

The reason for the higher production of KYNA in the presence of chestnut honey is unknown. Since honey is a complex mixture of different components, it can be speculated that currently unidentified compound(s) present in chestnut honey may stimulate yeast to produce KYNA. It should be also pointed out that the final produced yeast biomass in the medium containing chestnut honey was also higher (11.8 g/L) than in the culture on fructose (9.5 g/L).

The content of KYNA, determined in yeast biomass and culture broth in the current study, was much higher than measured for spices or food products (Table 2), for which the richest sources were basil and cacao powder [18,19]. KYNA was also found in potatoes in the range of 0.24–3.24 µg/g [20] and in fermented products such as red wine (in the range of 82.4–179.7 µg/L) and kefir (0.17 µg/g) [18]. Thus, the KYNA amount reported in present study is much higher than in all analyzed food or plant samples, except chestnut honey—for which a comparable KYNA level (129–601 mg/kg) was measured [10].

3.3. Analysis of Lipid Profile of Yeast Producing KYNA

The previous study showed the relationship between fructose concentration in medium and production of KYNA by yeast Y. lipolytica S12 [6]. The highest determined KYNA level was obtained in medium containing 40 g/L fructose and the detected concentration of KYNA was about 20-fold higher in culture broth and 24-fold higher in yeast biomass than in cultures performed in medium containing 80 g/L of fructose. At the same time, the highest biomass was observed for cultures with 80 g/L fructose and for these cultures, the biomass contained 36.9% of protein, while for cultures with 40 g/L of fructose, it was estimated that protein constituted 33% of the biomass. Since the excess of sugars can be redirected to the lipid metabolism, fatty acids composition of yeast cultured on chestnut honey and two concentrations of fructose (40 and 80 g/L) were analyzed and compared (Table 3).
Table 2. KYNA content in yeast biomass and broth of *Y. lipolytica* S12 strain, cultured in the medium with chestnut honey (50 g/L) in comparison to different food and plant samples.

| Product                          | KYNA Content (µg/g) | Reference |
|----------------------------------|---------------------|-----------|
| Yeast biomass (*Y. lipolytica* S12) | 542                 | present study |
| Culture broth (*Y. lipolytica* S12) | 67.67 *             | present study |
| Italian chestnut honey           | 576                 | [10]      |
| Basil (leaves)                   | 14.08               | [19]      |
| Thyme (leaves, branches)         | 8.87                | [19]      |
| Cacao powder                     | 4.48                | [18]      |
| Peppermint (leaves)              | 3.82                | [19]      |
| Parsley (leaves)                 | 0.76                | [19]      |
| Cumin (seeds)                    | 0.64                | [19]      |
| Potato tubers                    | 0.24–3.24           | [10]      |
| Kefir                            | 0.17                | [18]      |

Note: * µg/mL.

Table 3. Composition of fatty acids in the lipids produced in the culture of *Y. lipolytica* S12, containing different fructose amounts (40 or 80 g/L) or chestnut honey (50 g/L).

| Fatty Acid     | Lipid Number | 40 g/L Fructose A | 80 g/L Fructose B | 50 g/L Chestnut Honey C |
|----------------|--------------|-------------------|-------------------|-------------------------|
| Palmitic acid  | 16:0         | 9.98 ± 0.414 BC   | 8.17 ± 0.076 AC   | 26.93 ± 0.174 AB        |
| Palmitoleic acid | 16:1        | 5.47 ± 0.214 C    | 5.30 ± 0.131 C    | 12.52 ± 0.182 AB        |
| Stearic acid   | 18:0         | 7.11 ± 0.032 BC   | 5.57 ± 0.094 AC   | 14.41 ± 0.018 AB        |
| Oleic acid     | 18:1         | 33.13 ± 0.142 BC  | 55.54 ± 0.056 AC  | 31.18 ± 0.075 AB        |
| Linoleic acid  | 18:2         | 13.67 ± 0.165 C   | 13.10 ± 0.213 C   | 9.27 ± 0.143 AB         |
| Linolenic acid | 18:3         | 3.93 ± 0.045 B    | 1.14 ± 0.011 A    | n.d.                    |
| Gadoleic acid  | 20:1         | 5.01 ± 0.187 C    | 6.03 ± 0.076 C    | 2.28 ± 0.087 AB         |
| Erucic acid    | 22:1         | 5.47 ± 0.322 B    | 2.17 ± 0.151 A    | n.d.                    |
| Lignoceric acid| 24:0         | 10.83 ± 0.157 BC  | 1.57 ± 0.074 AC   | 3.36 ± 0.011 BC         |
| YL/X           | 7.47     | 8.75 | 4.73 |

Note: Fatty acids were analyzed as described in the Materials and Methods section via gas chromatography–mass spectrometry (GC–MS). YL/X—the lipid yield from biomass. Data are presented as %. n.d.—not determined. Statistical analysis was performed with one-way ANOVA with the Tukey post-test (GraphPadInStat). A,B,C p < 0.05. Capital letters in superscript A,B,C correspond to the respective columns.

As expected, the yeast cultured in medium containing 80 g/L fructose showed a higher concentration of lipids, i.e., 8.75% of dry weight. The lowest content of lipids was found in yeast growing on medium with chestnut honey.

The profile of fatty acids differed in the compared yeast cultures. The main fatty acids in the tested biomass obtained from medium with fructose were C16 and C18 and what is very interesting the sum of them was higher for cultures performed with a higher content of fructose; the sum of C16 and C18 was 88.62 and 73.29% for yeast cultured on 40 and 80 g/L of fructose, respectively. The main difference in the composition of fatty acids in the two types of biomass was the content of oleic acid (C18:1), which constituted 33.13% in cultures with a lower fructose content and 55.54% in biomass produced in yeast cultures with a higher fructose amount. The obtained results were comparable to Gao et al., 2020 [21]; however, in the cited article, the lipid content was much higher from 14.78 to 26.33%.

Additionally, the fatty acids composition was determined in the biomass derived from yeast cultured in media containing chestnut honey. It was found that the main fatty acid was oleic acid (C18:1), which constituted 31.18%, and palmitic acid (C16:0), which constituted 26.93%. The sum of C16 and C18 was equal to 94.31%, and linolenic acid (C18:3) and erucic acid (C22:1) were not detected.
3.4. Protein Content and Amino Acids Profile of Yeast Y. lipolytica S12 Producing KYNA

KYNA derives from tryptophan catabolism, thus it was reasonable to check the level of this amino acid as well as other amino acids in yeast producing KYNA. Results are depicted in Table 4.

### Table 4. Comparison of essential amino acid profiles and nutritional values of Y. lipolytica S12 protein.

| Amino Acid         | Honeydew Honey | French Chestnut Honey | Italian Chestnut Honey | Fructose 40 g/L + Trp 200 mg/L | Whole Egg 1 |
|--------------------|----------------|-----------------------|------------------------|-------------------------------|--------------|
|                    | A              | B                     | C                      | D                             |              |
| Isoleucine         | 1.42 ± 0.05 BCD | 3.36 ± 0.16 A         | 3.76 ± 0.17 AD         | 3.00 ± 0.10 AC                | 5.4          |
| Leucine            | 9.20 ± 0.13 BCD | 5.14 ± 0.18 AC        | 6.50 ± 0.21 ABD        | 5.65 ± 0.19 AC                | 8.6          |
| Lysine             | 4.65 ± 0.09 BCD | 3.45 ± 0.10 AC        | 3.93 ± 0.13 ABD        | 3.21 ± 0.09 AC                | 7.0          |
| Methionine/Cysteine| 2.68           | 2.43                  | 2.96                   | 2.10                          | 5.7          |
| Phenylalanine/Tyrosine| 4.12         | 4.73                  | 5.50                   | 3.90                          | 9.3          |
| Threonine          | 4.50 ± 0.11 BCD | 3.34 ± 0.11 A         | 3.40 ± 0.09 A          | 3.37 ± 0.13 A                 | 4.7          |
| Tryptophan         | 0.93 ± 0.02 BD  | 0.69 ± 0.02 AC        | 0.86 ± 0.03 ABD        | 0.64 ± 0.01 AC                | 1.7          |
| Valine             | 2.00 ± 0.04 BCD | 1.34 ± 0.05 A         | 1.43 ± 0.06 A          | 1.36 ± 0.04 A                 | 6.6          |
| Protein [%]        | 24.6          | 31.7                  | 33.3                   | 32.3                          |              |
| Nutritional values:|                |                       |                        |                               |              |
| CS iso-leucine     | 26.3          | -                     | -                      | -                             |              |
| CS valine          | -             | 20.3                  | 21.7                   | 20.7                          | -            |
| EAA                | 52.5          | 46.9                  | 54.0                   | 44.2                          | -            |
| ΣEAA               | 29.5          | 24.5                  | 28.3                   | 23.2                          | 49           |

Note: CS—chemical score—100× concentration ratio of the restrictive amino acid (ai)/concentration of this amino acid in the standard (a). ΣEAA—the sum of essential amino acids. EAAI—essential amino acid index. 1 FAO/WHO standard. The yeast biomass was obtained in cultures containing different carbon sources, i.e., different types of honeys (50 g/L) or 40 g/L fructose and 200 mg/L tryptophan. Data are presented as means ± SD. Statistical analysis was performed with one-way ANOVA with the Tukey post-test (GraphPadInStat). A,B,C,D p < 0.05. Capital letters in superscript A,B,C,D correspond to the respective column.

The lowest content of tryptophan in the biomass was found in cultures containing fructose (40 g/L) and supplemented with tryptophan (200 mg/L), which were the most effective conditions for KYNA synthesis in our previous study [6]. The content of tryptophan was significantly lower than in yeast cultured on honeydew and Italian but not French chestnut honey. Since production of KYNA in medium containing Italian and French chestnut honey was comparable (data not shown), it seems that the tryptophan level is not a critical factor.

The protein content was also estimated and the lowest contents of protein, reaching 24.6%, were determined in the biomass produced on the honeydew honey-containing medium (Table 4). For both chestnut honeys, the protein content in yeast biomass ranged from 31.7% to 33.3%. These cultures showed also the highest determined KYNA amount. It should be pointed out that protein content and amino acid profile are strongly dependent on the carbon source, the composition of the culture medium, culture conditions (e.g., cultivation time) the growth phase and used microorganism [22,23]. However, in the present study, the used yeast strain and conditions of all the cultures were the same, so the obtained differences are the results of the composition of the medium (type of honey or presence of fructose). Since the dry yeast biomass could potentially serve as a diet supplement, the biological value of the produced yeast protein was analyzed. Yeast biomass can provide valuable and alternative sources of protein exhibiting a nutritional value comparable to protein of animal origin [22–24]. In the analyzed yeast biomass, all essential amino acids were compared to the standard—whole egg protein (FAO/WHO standard) (Table 4). Obtained results are consistent with those exhibited for Y. lipolytica yeast, cultured in media containing waste products such as glycerol [2,25,26] or yeasts representing other species [27]. The content of amino acids in the yeast protein varied depending on the type of honey used as the carbon and energy source. The highest content of essential amino acids (ΣEAA = 29.5 and ΣEAA = 28.3) was found in the biomass, obtained in
the medium with honeydew and Italian chestnut honey, respectively. In the case of cultures performed in medium containing chestnut honey, the value of this parameter is related to the high content of leucine, at 6.5 g/100 g protein, phenylalanine and lysine, at 5.50 and 3.93 g/100 g protein, respectively. The lowest values of this index, $\Sigma_{\text{EAA}} = 23.2$ and $\Sigma_{\text{EAA}} = 24.5$, were calculated for yeast cultured in the medium with fructose (40 g/L) and French chestnut honey (Table 4). The higher amounts of essential amino acids were observed for $Y. \text{lipolytica}$ yeast strains cultured in the medium containing pure or crude glycerol [2,25,28]. Based on the amino acid composition, the limiting amino acid index chemical score (CS) and the essential amino acid index (EAAI) were calculated (Table 4). The low content of valine in the protein samples from cultures performed on French chestnut honey, Italian chestnut honey or fructose classifies it as a limiting essential amino acid. It means that a low content of valine limits the use of other exogenous amino acids for animals. However, in the available literature, the deficit of sulfuric amino acids (methionine, cysteine) in the yeast protein is widely discussed [24,28], because the content of them affects use in human and animal nutrition [29]. The biomass obtained in the present study, which is rich in sulfur amino acids, can be an excellent feed additive for animals.

The sum of essential amino acids in the examined yeast protein is lower in comparison to the standard—whole egg (Table 4). The highest value of the EAAI parameter was calculated for the yeast protein obtained in a medium with Italian chestnut honey and this parameter was comparable with values obtained for another strain of $Y. \text{lipolytica}$ (S6) in medium containing pure glycerol [25,28]. It should be also noted that for chestnut honey antibacterial and antioxidant properties have been found and described in the literature [30,31].

4. Conclusions

The present study provided new data related to the synthesis of bioactive compounds in yeast. The obtained results suggest that KYNA may be efficiently produced by the yeast $Y. \text{lipolytica}$ in media containing chestnut honey. Finally, the highest achieved concentration of KYNA was equal to 68 mg/L in culture broth and 542 mg/kg CDW in biomass. The determined KYNA amount in yeast biomass is comparable with the KYNA level in chestnut honey, which is the richest known source of this valuable compound. However, from an economic point of view, yeast biomass seems to be more efficient and it can be multiplied in large volumes in a short time and in controlled conditions.

The biological value of protein produced in yeast biomass was assessed and revealed the presence of essential amino acids. A raised essential amino acid index (EAAI) was noticed for biomass produced on honeydew honey and Italian chestnut honey. The last culture, which exhibited also the highest noticed KYNA amount, showed increased protein content when compared to the other analyzed yeast cultures.

Thus, it can be summarized that yeast biomass of $Y. \text{lipolytica}$ S12 may be considered as a new source of KYNA, the bioactive substance of medical significance. Further studies related to the feeding of animals with the produced yeast biomass, rich in KYNA, will be carried out in the near future.

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