Characterization and evaluation of coconut aroma produced by \textit{Trichoderma viride} EMCC-107 in solid state fermentation on sugarcane bagasse

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**A R T I C L E   I N F O**

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**A B S T R A C T**

**Background:** Sugarcane bagasse was shown to be an adequate substrate for the growth and aroma production by \textit{Trichoderma} species. In the present work the ability of \textit{Trichoderma viride} EMCC-107 to produce high yield of coconut aroma in solid state fermentation (SSF) by using sugarcane bagasse as solid substrate was evaluated. The produced aroma was characterized.

**Results:** Total carbohydrates comprised the highest content (43.9% w/w) compared with the other constituents in sugarcane bagasse. The sensory and gas chromatography–mass spectrometric (GC–MS) analysis revealed that the highest odor intensity and maximum yield of volatiles were perceived at the 5th d of induction period. The unsaturated lactone, 6-pentyl-α-pyrone (6-PP), was the major identified volatile compound. Saturated lactones, δ-octalactone, γ-nonalactone, γ-undecalactone, γ-dodecalactone and δ-dodecalactone, were also identified in the coconut aroma produced during the induction period (12 d). A quite correlation was found between the composition and odor profile of the produced aroma. The effect of varying the concentration of sugarcane bagasse on 6-PP production and biomass growth was evaluated. The results revealed high 6-PP production at 4.5 g sugarcane bagasse whereas the biomass showed significant ($P < 0.05$) increase by increasing the concentration of sugarcane bagasse.

**Conclusion:** The concentration of 6-PP, the most contribution of coconut aroma, produced in present study (3.62 mg/g DM) was higher than that reported in previous studies conducted under the same fermentation conditions. The significant increase in biomass with increasing the concentration of sugarcane bagasse may be attributed to the increase in sugar content that acts as carbon and energy source.

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1. Introduction

In recent years the consumers’ demand for natural food additives is rising. The food and drug administration \cite{1} stated that to qualify as ‘natural’, flavorings have to be produced by physical, enzymatic or microbiological processes from natural sources. The Food and Drug Administration (FDA) defines natural aroma and specifies the type of substances generally regarded as safe for use as natural flavors including any substance that is extracted, distilled, or otherwise derived from plant or animal matter. Microorganisms play an important role in the generation of natural compounds, particularly in the field of food aromas. Several reports and reviews have been published on the production of volatile compounds (aroma compounds) by microorganisms \cite{2,3,4,5,6,7,8,9}. Although several bacteria, yeast and fungi have been reported for the production of aroma compounds, a few species of yeasts and fungi have generally been preferred, and only a few of them find industrial application due to their generally regarded as safe (GRAS) status. Nowadays, there has been an increase trend toward the more efficient use of agro-industrial wastes including sugarcane bagasse \cite{10,11}. Wastes from the food and agricultural industries that are produced in large quantities and are rich in carbohydrates and other nutrients can serve as a substrate for the production of chemicals and enzymes by using the technique of solid state fermentation (SSF), mainly due to their low cost \cite{12}. In Egypt, the processing of the high production of sugarcane (15.77 million tons, Ministry of Agriculture, 2011–2012) gives rise to a large amount of sugarcane bagasse whose accumulation leads to an important problem of environmental pollution. SSF has been used for the production of aroma compounds by cultivating fungi using agro industrial residues as substrates \cite{11}. The nature of the solid substrate used is an important aspect. However, the solid substrate not only supplies nutrients for microbial culture, but also serves as the physical support for the growth of microbial cells. Production of coconut aroma by \textit{Trichoderma} species in SSF have been...
investigated in previous studies [11,13,14]. Trichoderma species are reported as good producers of the unsaturated δ-lactone 6-pentyl-α-pyrene (6-PP) [15,16]. This compound is of interest in the food industry; it was found to be the major volatile compound contributing to the coconut-like aroma in cultures of Trichoderma viride [17]. 6-PP is approved as a flavoring agent by Joint Expert Committee on Food Additive [16]. Fungicide properties of 6-PP were also reported [16]. Ramos et al. [13] and Penha et al. [11] stated that the biosynthesis of 6-PP by Trichoderma species in SSF was accompanied by the production of other compounds. However, the authors of these studies didn’t identify these compounds. T. viride EMCC-107 was recorded only in Egypt by the Microbiological Resources Center. This strain was used for bio-treatment of agro industrial wastes [18] but to the best of our knowledge it was not used for aroma production. Therefore, in this study the ability of T. viride EMCC-107 to produce high yield of coconut aroma in SSF by using sugarcane bagasse as substrate was explored. Characterization of the volatile compounds accompanied the 6-PP production and evaluation of their influence on the overall odor profile of the aroma produced during the period of fermentation was estimated. The study was extended to evaluate the effect of varying the level of sugarcane bagasse on culture growth and aroma production. Finally, the relationship between the sensory profile and composition of the produced aroma was investigated.

2. Materials and methods

2.1. Microbial strain and culture media

The T. viride EMCC-107 purchased from the collection of Microbiological Resources Center (MIRCEN, Egypt) was used. The strain was maintained on malt extract medium at 4°C and cultured in Petri dishes at 28°C. A 5 d-old mycelium obtained under these conditions was used to inoculate the liquid media. The latter medium (50 mL), containing malt extract (20 g/L) and glucose (10 g/L), pH 5.6, was placed into 250 mL Erlenmeyer flasks and autoclaved at 121°C for 20 min before inoculation with 1 mL of mycelium grown on malt extract medium. The flasks were incubated at 28°C for 72 h on orbital rotatory shaker (100 rpm). The mycelium formed was separated by decantation and rinsed twice with physiological saline solution (0.9% NaCl). It was then suspended into 50 mL of the same saline solution and 2 mL of this suspension were used to inoculate the solid substrates used.

2.2. Substrate preparation

Sugarcane bagasse was obtained from a local market for the production of sugarcane juice in Egypt. The bagasse was subjected to a series of operations, comprising: (i) grinding with a mill using knives and hammer (1 mm), (ii) drying at 60°C for 24 h and ground in a granulator mill using knives and hammer, and (iii) autoclaving at 121°C for 20 min. Each sample was freeze-dried for 12 h and used for extraction.

2.3. Chemical composition of sugarcane bagasse

The chemical constituents of sugarcane bagasse were determined as described by AOAC [19]. Fatty acid composition of the lipid fraction was determined according to AOAC [20].

2.4. Fermentation conditions

Fermentation process was conducted for 12 days and analyzed for the production of coconut aroma at the 3, 5, 7, 9, 12th d. The solid substrate, comprising 4.5 g sugarcane bagasse was placed into 500 mL conical flask and autoclaved at 121°C for 20 min. Each flask was impregnated with 25 mL of sterile medium containing (g/L) glucose, 30.0; (NH₄)₂SO₄, 0.94; KH₂PO₄, 7.0; Na₂HPO₄.7H₂O, 2.0; MgSO₄.7H₂O, 1.5; CaCl₂.2H₂O, 0.008; FeCl₃.6H₂O, 0.008; ZnSO₄.7H₂O, 0.0001 [14]. The flasks were inoculated with 1 mL of mycelia cell suspension and incubated at 28°C. The same conditions of SSF that revealed the high quality of coconut flavor and optimum yield of 6-PP (the most contributor of coconut aroma) was carried out but with variable levels of sugarcane bagasse (1.5, 3.0, 4.5 and 6.0 g/500 mL conical flask).

2.5. Dry matter measurement

Dry matter (DM) was determined by weight differences, 2–3 g fermented substrate was weighed and then dried to constant weight at 105°C.

2.6. Sensory evaluation

The effect of incubation time on the odor profile and intensity of the perceived odor of T. viride EMCC-107 culture in SSF on sugarcane bagasse was investigated. A panel of 10 members (National Research Center, Cairo, Egypt) characterized the odor of the fungal culture on the 3rd, 5th, 7th and 12th d. The odor description was assessed and the intensity was estimated on a 4 point scale (+: weak odor, ++: medium odor, ++++: strong odor, ++++:+: very strong odor). The time of incubation period that revealed the optimum quality of coconut odor was selected to estimate the effect of varying the level of sugarcane bagasse on the intensity of the perceived odor. A nine-point hedonic scale (1 = not perceptible to 9 = strong perceptible) was used. Samples were identified with three digit code numbers and presented in a random sequence to the panelists.

2.7. Extraction of aroma compounds

Sample (4.5 g) was removed from each solid state culture and placed into 250 mL flask with 50 mL distilled water. The aroma compounds were extracted from the samples with 50 mL dichloromethane.

2.8. Gas chromatographic (GC) analysis

GC analysis was performed by using the Hewlett-Packard model 5890 equipped with flame ionization detector (FID). A fused silica capillary column DB5 (60 m x 0.32 mm, i.d.) was used. For fatty acid analysis, the oven temperature was programmed from 50 to 240°C at a rate of 3°C/min. Helium was used as the carrier gas, at flow rate 1.1 mL/min. The injector and detector temperatures were 220 and 250°C, respectively. For analysis of the extracted coconut aroma, the oven temperature was maintained initially at 50°C for 6 min, and then programmed from 50 to 240°C at a rate of 3°C/min. The injector and detector temperatures were 220 and 240°C, respectively. The retention indices (Kovats index) of the separated volatile compounds were calculated with hydrocarbons (C8–C22, Aldrich Chemical Co.) as references. The relative concentration of each individual compound was determined by comparing the peak area of the compound in each chromatogram with that of γ-decalactone (IS), assuming all response factors were 1.

2.9. Gas chromatographic–mass spectrometric (GC–MS) analysis

The analysis was carried out using a coupled gas chromatography Hewlett-Packard 5890/mass spectrometry Hewlett-Packard–MS 5970). The ionization voltage was 70 eV, mass range m/z 39–400 amu. The GC condition carried out as mentioned above. The isolated peaks were identified by matching with data from the library of mass spectra.
used as a carbon energy source by previous studies [11]. The fatty acid composition of the lipid fraction (0.20% w/w) was lower than that reported (1.36 and 0.7% w/w) in favor of 6-PP biosynthesis. In the present study the level of lipid non-hydroxylated e.g. oleic, linoleic and linolenic fatty acids [16]. It is possible to produce 6-PP from different vegetable oils containing 21.04 ± 0.51% and linolenic, 4.05 ± 0.36%. It was demonstrated that it was palmitic, 33.64 ± 0.23%; palmitoleic 5.34 ± 1.62%; heptadecanoic, 7.78 ± 0.48%; stearic, 5.06 ± 0.23%; oleic, 23.06 ± 0.60%; linoleic, 21.04 ± 0.51% and linolenic, 4.05 ± 0.36%. It was demonstrated that it is possible to produce 6-PP from different vegetable oils containing non-hydroxylated e.g. oleic, linoleic and linolenic fatty acids [16]. The total carbohydrates comprised the highest content (43.9% w/w) compared with other constituents in sugarcane bagasse in present study. It is higher than that reported in previous literatures [11]. Sugars used as a carbon energy source by Trichoderma sp. for the production of 6-PP at the same efficiency as that with fatty acids [16].

3. Results and discussion

3.1. Chemical composition of sugarcane bagasse

Production of the volatile compounds by fungi in SSF can be influenced by the type and constituents of the substrate used that act as carbon and energy sources [6,23]. In the present study the level of moisture (11.3% w/w) in dried sugarcane bagasse may be correlated to the processing methods used during its preparation. In SSF the fermentation take place in the absence or near absence of free water, thus being close to the natural environment to which microorganisms are adapted [10]. The protein content (1.9% w/w) in dried sugarcane bagasse was as low as that detected in previous study [11]. The effect of culture parameters and choice of precursors for 6-PP biosynthesis was studied [24]. The authors concluded that a low nitrogen level was favorable for 6-PP biosynthesis. In the present study the level of lipid (0.20% w/w) was lower than that reported (1.36 and 0.7% w/w) in previous studies [11]. The fatty acid composition of the lipid fraction was palmitic, 33.64 ± 0.23%; palmitoleic 5.34 ± 1.62%; heptadecanoic, 7.78 ± 0.48%; stearic, 5.06 ± 0.23%; oleic, 23.06 ± 0.60%; linoleic, 21.04 ± 0.51% and linolenic, 4.05 ± 0.36%. It was demonstrated that it is possible to produce 6-PP from different vegetable oils containing non-hydroxylated e.g. oleic, linoleic and linolenic fatty acids [16]. The total carbohydrates comprised the highest content (43.9% w/w) compared with other constituents in sugarcane bagasse in present study. It is higher than that reported in previous literatures [11]. Sugars used as a carbon energy source by Trichoderma sp. for the production of 6-PP at the same efficiency as that with fatty acids [16].

3.2. Dry matter (DM)

The DM of the fermented substrate showed significant (P < 0.05) increases by increasing the incubation period up to 7 d followed by insignificant (P > 0.05) increase (Fig. 1). This result is in agreement with that of De-Araujo et al. [14]. The toxic effect of 6-PP, the major volatile compound produced during fermentation, toward the fungal growth was demonstrated in previous studies [16]. The high production of 6-PP after 5 d of incubation in present study (as will be discussed below) may be transformed into other compounds. This transformation probably serves to avoid the toxic effect that this molecule has on the growth of Trichoderma species [25]. The effect of varying the concentration of sugarcane bagasse on the culture growth is shown in Fig. 2. It is obvious that increasing the level of sugarcane bagasse in SSF media gave rise to a significant (P < 0.05) increase in the biomass production. This finding may be correlated to the increase in the quantities of sugar and oil, in the solid substrate, that can be acted as a carbon and energy sources. Bonnarme et al. [16] demonstrated that oils and sugars promote biomass and 6-PP accumulation and at the same time the oil acts as a detoxifying agent against the toxicity effect of the high production of 6-PP.

3.3. Sensory evaluation

The effect of fermentation time on the characteristics of the perceived aroma and the total released volatiles produced by T. viride EMCC-107 in SSF on sugarcane bagasse are shown in Table 1. Strong coconut aroma was perceived after incubation for 5 d. The intensity of coconut aroma decreased after 7 d. After 9 d the odor was rather weak coconut/fruity. Whereas, after 12 d odor of the culture possessed slight sweet fruity aroma. It is obvious that, the aroma intensity was correlated with the yield of total volatiles detected by GC analysis.

The effect of varying the concentration of sugarcane bagasse on the intensity of coconut aroma was investigated on the 5th d of fermentation which occurring commonly in soil

3.4. Analysis of aroma compounds

3.4.1. Effect of time of fermentation on aroma production

A total of six volatile compounds were identified in the volatiles extracted from the SSF culture of T. viride EMCC-107 during the incubation period (12 d). Table 2 shows the recovered amount of each compound as well as the description of its odor as reported in literatures. 6-PP, the potent aroma compound in coconut aroma, was the major compound after incubation for 5 d. It comprised more than 98% of all identified compounds as observed by GC–MS analysis. 6-PP is known as one of the major volatile compound biosynthesized by certain species of Trichoderma fungi which occurring commonly in soil.
Table 1

<table>
<thead>
<tr>
<th>Fermentation time (d)</th>
<th>Aroma description</th>
<th>Aroma intensities</th>
<th>Total volatiles (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Fermented note</td>
<td>++</td>
<td>0.25</td>
</tr>
<tr>
<td>5</td>
<td>Coconut aroma</td>
<td>+++++</td>
<td>17.55*</td>
</tr>
<tr>
<td>7</td>
<td>Coconut aroma</td>
<td>+++++</td>
<td>16.18a</td>
</tr>
<tr>
<td>9</td>
<td>Coconut/fruity</td>
<td>+</td>
<td>3.10a</td>
</tr>
<tr>
<td>12</td>
<td>Sweet/fruity</td>
<td>+</td>
<td>0.41</td>
</tr>
</tbody>
</table>

Values are the average of triplicate analysis, mg/g dry matter of fermented substrate. Mean values in the same row followed by different superscript letters are significantly different (P < 0.05).

Table 2

<table>
<thead>
<tr>
<th>Volatile compounds</th>
<th>RI*</th>
<th>Time of fermentation (d)</th>
<th>Aroma description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Octalactone</td>
<td>1290</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>γ-Nonalactone</td>
<td>1369</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td>γ-Dodecalactone</td>
<td>1765</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>δ-Dodecalactone</td>
<td>1800</td>
<td>12</td>
<td>-</td>
</tr>
</tbody>
</table>

Values are the average of triplicate analysis, mg/g dry matter of fermented substrate. Mean values in the same row followed by different superscript letters are significantly different (P < 0.05).

4. Conclusion

The production of 6-PP by *T. viride EMCC-107* in SSF on sugarcane bagasse after 5 d of fermentation was higher (3.62 mg) than that reported in previous studies using the same substrate and fermentation conditions. The identification of the saturated γ- and δ-saturated lactones accompanied with 6-PP production confirmed the changes in aroma profile during the induction period (12 d). Variation in the level of sugarcane bagasse influenced the volatile production and culture growth. The positive correlation between the level of sugarcane bagasse and biomass production was attributed to the total sugar content that acts as carbon and energy source. In general using sugarcane bagasse as solid substrate in SSF for high 6-PP production by *T. viride EMCC-107* has an economic and environmental benefits.
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References