Biocatalytic Preparation of Natural Flavours and Fragrances

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## Total Global Consumption of Flavours & Fragrances by Region, 2015-2020 (US$ Millions)

<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Africa &amp; Middle East</td>
<td>1,330.40</td>
<td>1,677.45</td>
<td>4.7%</td>
</tr>
<tr>
<td>Asia</td>
<td>8,533.72</td>
<td>12,188.95</td>
<td>7.4%</td>
</tr>
<tr>
<td>Central &amp; North America</td>
<td>5,994.43</td>
<td>7,423.27</td>
<td>4.4%</td>
</tr>
<tr>
<td>Central &amp; Eastern Europe</td>
<td>1,521.28</td>
<td>1,776.00</td>
<td>3.1%</td>
</tr>
<tr>
<td>South America</td>
<td>1,774.19</td>
<td>2,240.81</td>
<td>4.8%</td>
</tr>
<tr>
<td>Western Europe</td>
<td>4,413.29</td>
<td>4,893.21</td>
<td>2.1%</td>
</tr>
<tr>
<td><strong>Global Total</strong></td>
<td><strong>23,567.32</strong></td>
<td><strong>30,199.69</strong></td>
<td><strong>5.1%</strong></td>
</tr>
</tbody>
</table>
Global Market For Flavours 2013

- Bakery
- Beverages
- Confectionery
- Dairy
- Meat
- Oral Hygiene/Pharmaceutical
- Other Flavours
- Savoury/Convenience
- Snacks
INTRODUCTION

Global Market For Fragrances 2013

- Cosmetics & Toiletries
- Fine Fragrances
- Household Cleaners & Air Fresheners
- Other Fragrances
- Soap & Detergents

http://www.ialconsultants.com
Vanilla is a complex blend of flavour and fragrance ingredients extracted from the seed pods of the vanilla orchid. The most important ingredient in this blend is vanillin.

99% of all vanillin consumed worldwide made primarily from petrochemicals or chemically derived from lignin.

Sales prices range from about USD 1,500 per kg for natural vanilla extract to USD 10-20 per kg for synthetic vanillin.

Natural vs Synthetic of Flavours and Fragrances

**Natural**

- Obtained from plant or animal sources, by physical, microbiological, or enzymatic processes
- Are typically complex mixtures of chemicals
- Consumer-friendly
- Exhibit variations in strength & quality

**Synthetic**

- Synthesized from other chemicals
- Usually contain only a small number (often just one) of the compounds, and lack the others so they cannot precisely duplicate the flavor of the complex mixture
- Consumer-unfriendly
- Consistency in quality

Production of Flavours & Fragrances

- Plants naturally
  - low concentrations
  - environment dependent
  - The isolation of active compounds is difficult

- Chemical synthesis
  - environmentally unfriendly
  - poor reaction selectivity
  - high manufacturing and costs

- Biological synthesis
  - renewable resources
  - clean production
  - less pollution
  - less energy intensive processes
Production of Flavours & Fragrances

Plants naturally

Ethyl butyrate
C₆H₁₂O₂

cinnamon bark

Cinnamaldehyde
C₉H₈O

Citronellol
C₁₀H₂₀O

https://www.google.com.tw
Production of Flavours & Fragrances

Industrial route of vanillin production from phenol

Phenol

\[ \text{H}_2\text{O}_2 \xrightarrow{\text{H}^+} \text{Guaiacol} \]

\[ \text{Me}_2\text{SO}_4 \xrightarrow{} \text{Vanillin} \]

\[ \text{KOH} \quad \text{Vanilglycolic acid} \]

\[ \text{Cu(II)} \quad \text{Vanillylmandelic acid} \]

Production of Flavours & Fragrances

Biological synthesis

Esters
(fruity flavors and aromas in wine)

**Biological synthesis**

- De novo synthesis
  - the production of aroma compounds after metabolizing cells by using simple cultivation media

**Advantages & disadvantages**

- ✓ can use cheap raw materials, ex. simple sugar
- ✓ produces a mixture of several aroma compounds
- ✓ low concentrations of produced aroma compounds

**Biotransformation**

- the use of microbial cells or enzymes to perform specific transformation of precursor into desired product

**Advantages & disadvantages**

- ✓ need precursor
- ✓ leads to one major product produced by specific conversion
- ✓ high concentrations of produced aroma compounds

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Akachaa & Gargouri. Food Bioprod Process 2014;94:675-706
De Novo Engineered De Novo Vanillin Biosynthesis from Glucose in the Yeasts S. cerevisiae and Schizosaccharomyces pombe.

D-glucose → 3-dehydroshikimic acid → Protocatechuic acid

Protocatechuic aldehyde → Vanillic acid

Vanillin

Gallage and Møller, Mol Plant 2015;8:40-57
Biotransformation

Metabolic pathways of ferulic acid in different strains

Different Routes to Natural Vanillin that Are Available or Soon to Be Available on the Market

- **Rice bran**
  - Ferulic acid
  - **Bioconversion by fermentation**

- **Vanilla pods**
  - Extraction
  - **Soft chemistry & fermentation**

- **Clove**
  - Eugenol
  - **Bioconversion by fermentation**

- **Corn**
  - Glucose
  - **De novo biosynthesis by fermentation**

- **Turmeric**
  - Curcumin
  - **Bioconversion by fermentation**
Natural Flavors Prepared by Microbiological Methods

Generation of aroma compounds by microbial methods

De novo synthesis

Biotransformation

Submerged fermentation

Solid-state fermentation

Akacha & Gargouri. Food Bioprod Process 2014;94:675-706
Generation of aroma compounds by microbial methods

Advantages & Disadvantages

- Easy to scale up
- High mix effectively
- Easy to control
- Lower impurity product
- Generally, lower fermentation productivity
- High production costs

Submerged fermentation

Substrate
- Eugenol
- Lignin

Microorganisms
- *Pseudomonas sp.*
- *Aspergillus niger*
- *Corynebacterium strains*
- *Pleurotus cornucopia*
- *Pleurotus eryngii*
- *Pleurotus floridanus*
- *Pleurotus pulmonarius*

Flavor compounds
- Vanillin
- Anisaldehyde

References
Akacha & Gargouri. Food Bioprod Process 2014;94:675-706
Generation of aroma compounds by microbial methods

Substrate
- Coffee husk
- Soybeans

Micro-organisms
- Ceratocystis fimmbriata
- Kluyveromyces marxianus
- Bacillus natto
- Bacillus subtilis

Flavor compounds
- Ethyl acetate
- Pyrazine

Advantages & Disadvantages
- Higher fermentation productivity
- Extended stability of products
- Low production costs
- Lower energy and low water demand
- Difficult control of process parameters
- Difficulties on scale up

Solid-state fermentation

Akacha & Gargouri. Food Bioprod Process 2014;94:675-706
Examples of flavor compounds obtained from microbial *de novo synthesis*

<table>
<thead>
<tr>
<th>Flavours</th>
<th>Sensorial description</th>
<th>Fungi</th>
<th>Yeast</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-phenylethanol</td>
<td>Rose-like aroma</td>
<td>Pleurotus sapidus</td>
<td><em>S. cerevisiae</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Polyporus sp.</td>
<td><em>K. marxianus</em></td>
</tr>
<tr>
<td>Geraniol</td>
<td>Sweet, rose-like, fruity</td>
<td><em>C. moniliformis</em></td>
<td><em>K. lactis</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>C. variospora</em></td>
<td></td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>Bitter almond</td>
<td><em>Ischnoderma benzoinum</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Agaricus subrufecens</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Agaricus bisporus</em></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td><em>Armillaria mellea</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Polyporus sp.</em></td>
<td></td>
</tr>
</tbody>
</table>

Akacha & Gargouri. Food Bioprod Process 2014;94:675-706
Examples of flavor compounds obtained from microbial transformations

<table>
<thead>
<tr>
<th>Flavours</th>
<th>Metabolic intermediates</th>
<th>Biocatalysts</th>
<th>Microorganisms</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzaldehyde</td>
<td>2-phenylethanol</td>
<td>Phenylpyruvic acid</td>
<td>Transaminase</td>
<td>Polyporus tuberaster</td>
</tr>
<tr>
<td>or</td>
<td>Phenylacetaldehyde</td>
<td>Decarboxylase</td>
<td>I. benzoinum</td>
<td>Krings et al. (1996)</td>
</tr>
<tr>
<td>2-phenylethanol</td>
<td>Phenylalanine</td>
<td>I. benzoinum</td>
<td>B. adusta</td>
<td>Lapadatescu et al. (1999)</td>
</tr>
</tbody>
</table>

K. marxianus, S. cerevisiae.

Krings et al. (1996), Fabre et al. (1998), Stark et al. (2002).

Akacha & Gargouri. Food Bioprod Process 2014;94:675-706
Examples of flavor compounds obtained from enzymatic biotransformations

<table>
<thead>
<tr>
<th>Flavors</th>
<th>Flavoring types</th>
<th>Enzyme-catalysed reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Z)-3-hexenol</td>
<td>Grassy, green odor</td>
<td>Bioconversion of hydrolyzed linseed oil in enzymatic liquid/gas reactor by sequential action of soybean lipoxygenase, hydroperoxide lyase from olive leaves and alcohol dehydrogenase from baker’s yeast</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2E,6Z-nonadienal</td>
<td>Cucumber or watermelon</td>
<td>Bioconversion of mixture of polyunsaturated fatty acids by sequential action of lipoxygenase and hydroperoxide lyase from viola leaves</td>
</tr>
</tbody>
</table>

Akacha & Gargouri. Food Bioprod Process 2014;94:675-706
Engineering Challenge

Production

Cytotoxicity

Downstream processing

Others
**Production**

**Pretreatment of raw materials**

The pretreatment of **cane molasses** using **sulfuric acid** increased the concentration of butyrate of 55.2 g/L using **Clostridium tyrobutyricum** (Jiang et al., 2009)

**Optimization of medium composition & culture conditions**

- medium formulation
- temperature
- pH
- aeration, etc.

**Design of bioreactor**

- Stirred tank
- Packed-bed
- Fluidized bed

**Operation mode**

- Batch
- Fed-batch
- Continuous
- Semi-continuous

**Genetic engineering**

Have a potential however the application remains sensitive due to the complexity of cellular potential and of regulation in the host microorganism
Cytotoxicity

Have to be removed from the fermentation broth due to can give an inhibitory effect to the cells.

Nutrients

Cell

Intrinsic metabolism

Hydrophobic Aroma Compounds

Fermentor

In situ product removal (ISPR) is a powerful tool to overcome this limitation.
Cytotoxicity

In Situ Product Removal Methods

- Organophilic pervaporation
- Supercritical CO$_2$ extraction
- Solvent immobilization
- Adsorption
- Two-phase extraction
Downstream Processing

Primary Recovery

High volatility and low solubility in water

Purification

selective product recovery

Finishing

Crystallization, packaging, etc.
Study of behavior of volatile compounds between phases

Chiral separation of racemic mixtures of aroma compounds
Study of behavior of volatile compounds between phases

$C_L$: concentration in the liquid phase; $C_G$: concentration in the headspace; $P_{GL}$: gas-liquid partition coefficient; $\delta_G$: the thickness of the gas layer; $\delta_L$: the thickness of the liquid layer

Schematic diagram of flavour concentrations at the liquid-gas interface during release from the liquid phase
Many components of natural products can be chiral, which leads to the formation of one pair of enantiomers. These compounds have identical physical and chemical properties but show different odor or other properties.
Chiral separation of racemic mixtures of aroma compounds

Racemic of 4-Methyloctanoic acid

Natural occurrence: Cooked mutton fat, raw and cooked mutton, some kinds of cheeses

(R)-(-)-4-Methyloctanoic acid

More goaty type character, typical mutton taste and smell, sweeter, 5 to 10 times stronger than S-enantiomer

(S)-(+)-4-Methyloctanoic acid

Less goaty type character, fatty, less interesting than R-enantiomer

Other

Chiral separation of racemic mixtures of aroma compounds

Kinetic resolution of racemic 4-methyloctanoic acid

4-methyloctanoic acid + EtOH

*Candida antarctica* lipase B

\[ \text{R-Ester} + \text{H}_2\text{O} \rightarrow \text{S-acid} \]

BIOCONVERSION OF PHENYLALANINE TO 2-PHENYLETHANOL
Most of which was obtained by chemical synthesis from benzene or styrene with a price of about US$ 5/kg.

2-Phenylethanol (PEA)

Aromatic alcohol with a rose-like fragrance

It is widely applied in the cosmetics, perfumery, pharmaceutical and food industries.

Natural PEA can be extracted from the essential oils of certain flowers (e.g., rose). However, the concentration of 2-PE in flowers is very low, and the extraction process is therefore complicated and costly (about US$ 1000/kg).

Hua D, Xu P. Biotechnol Adv 2011;29:654-60
De Novo Glucose Glycolysis
Erythrose-4-phosphate

Pentose Phosphate Pathway
Phosphoenol pyruvate

Shikimate
Chorismate
Prephenate

transaminase
L-Phenylalanine
Phenylpyruvate
Phenylacetaldehyde
PEA

Shikimate pathway for de novo synthesis of 2-phenylethanol (PEA)

Hua D, Xu P. Biotechnol Adv 2011;29:654-60
Bioconversion

Ehrlich pathway for bioconversion of L-Phe to PEA

Hua D, Xu P. Biotechnol Adv 2011;29:654-60
Microorganisms as PEA Producers

Saccharomyces cerevisiae
Kluyveromyces spp.

Pichia membranaefaciens

Pichia anomala

Yarrowia lipolytica

Kloeckera saturnus

Hansenula anomala

Zygosaccharomyces rouxii

Clavispora lusitaniae

Morrissey et al. Yeast 2015; 32: 3-16
Several possible mechanisms of inhibitory actions of PEA

- Increase the membrane fluidity hence cause leakage of ions and reduce uptake of amino acids and glucose.
- Induce a respiratory deficiency hence inhibit the growth of \textit{S. cerevisiae}.
- Increased mitochondrial permeability was proposed as the reason inhibition of respiration.
- PEA has also been investigated as a bacterial inhibitor of macromolecular synthesis.

\footnotesize{Stark et al. Enzyme Microb Technol 2003;32:212-23}
Experimental Design

In this study, the optimum condition of culture on the bioconversion of L-Phe to PEA using *Saccharomyces cerevisiae* BCRC 21812 was investigated and the development of strategies in order to enhance the concentration of PEA was also conducted.
Methods

2 loops

30 °C and 120 rpm for 24 h

10 ml

30 °C and 120 rpm for 12 h

S. cerevisiae BCRC 21812

Air in

Pump

Rotameter

100 ml

100 ml

20 ml

Water

Bioreactor

Water Bath
The Optimization of culture conditions

1. Effect of aeration and L-Phe concentrations on the biomass concentration and the biomass productivity

2. Effect of air flow rate on the biomass concentration and the biomass productivity

3. Inhibition effects of PEA and ethanol on the bioconversion
Effect of aeration condition on the biomass growth and the biomass productivity

- Biomass (g/L)
- PEA (g/g DW)
Effect of L-Phe concentrations on the biomass growth and the biomass productivity
Effect of air flow rate on the biomass concentration and the biomass productivity
The inhibition effect of ethanol on specific growth rate of *S. cerevisiae* at various exogenous ethanol concentration.
The inhibition effect of ethanol on specific growth rate of *S. cerevisiae* at various exogenous ethanol concentration.

\[
y = -0.05011x + 2.05824 \quad \text{0.1 vvm}
\]

\[
y = -0.04561x + 2.35532 \quad \text{0.4 vvm}
\]

Graph showing the relationship between specific growth rate and exogenous ethanol concentration.
The inhibition effect of PEA on specific growth rate of *S. cerevisiae* at various exogenous PEA concentration

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**Diagram:**

- **Y-axis:** \( \mu \) (d\(^{-1}\))
- **X-axis:** Exogenous PEA Concentration (g/L)
- Data points for 0.1 vvm and 0.4 vvm at concentrations 0, 0.5, 1.5, and 2.5 g/L
The inhibition effect of PEA on specific growth rate of *S. cerevisiae* at various exogenous PEA concentration

\[ y = -0.87323x + 2.19625 \quad \text{0.1 vvm} \]

\[ y = -0.59935x + 2.24999 \quad \text{0.4 vvm} \]
Two-stages fermentation for the bioconversion of L-Phe to PEA using S. cerevisiae BCRC 21812

0.4 vvm  0.1 vvm
Comparison between single stage and two-stages fermentation in bioconversion of L-Phe into PEA using *S. cerevisiae* BCRC 21812
Strategies enhancing bioconversion

1. The semi-continuous operation mode

2. The semi-continuous and ISPR using PDMS sponge

3. Three stages of fermentation combined with the semi-continuous operation mode and ISPR
The semi-continuous bioconversion of L-Phe to PEA without ISPR

Total PEA = 2.25 g/L
In Situ Product Recovery (ISPR) using PDMS sponge

PDMS sponge

Sterile Water
Fermentation medium
Sponge
The semi-continuous bioconversion of L-Phe to PEA with ISPR using PDMS sponge

Total PEA = 3.05 g/L or 35.6% higher than without ISPR for the same fermentation time
Three stages of fermentation combined with the semi-continuous operation mode and ISPR using PDMS sponge

Total PEA = 5.14 g/L or enhanced 39.3% than two-stages with ISPR
References

Thank you