

MEDIA COMPONENTS AND AMINO ACID SUPPLEMENTS INFLUENCING THE PRODUCTION OF FRUITY AROMA BY *GEOTRICHUM CANDIDUM*

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SHORT COMMUNICATION

ABSTRACT

The ability of *Geotrichum candidum* to produce fruity aroma in food grade sucrose, molasses, corn steep liquor and peptone based culture media was tested by sensory evaluation and analyzed by gas chromatography – mass spectrometry. A strong and sweet fruity aroma was produced from molasses, with peptone or corn steep liquor stimulating aroma production. Molasses with peptone supplemented with leucine, valine, or alanine yielded better fruity aroma production and the presence of many esters was consistent with the fruity aroma production.

Key words: *Geotrichum candidum*, aroma, corn steep liquor, molasses, amino acids

Flavors and fragrances are important to the food, cosmetic, pharmaceutical and chemical industries. According to Leffingwell and Associates (http://www.leffingwell.com/top_10.htm), the world market size was about US\$ 17.5 billion in 2004. Although chemical synthesis remains important, there has been a trend in consumer preference toward natural flavors (6) motivating the biotechnological development of aromas. Natural aroma production can include enzymes, plant cell cultures and microorganisms (10). Some microbes are known to produce aromas in laboratory scale cultures. However, for industrial scale production costs are an important factor so the use of inexpensive culture media is essential. The addition of precursors which stimulate the aroma production should help solve this problem.

Geotrichum candidum is a yeast-like fungus used for commercial cheese ripening. Enzymatic activities from lipases, proteinases and peptidases modify the appearance, texture and flavors of cheeses. *G. candidum* makes a significant contribution to the distinctive flavor and appearance for Camembert, Pont

l'Evêque, Münster, Limburger, Livarot, Saint Nectaire and Reblochon cheeses (3,4,5,11). Some strains may produce esters, often related to specific fruit aroma but only a few studies have used this microorganism to produce fruity aroma (9,10,20). These studies have shown that *G. candidum* is variable in its ability to produce aroma, depending on differences in culture media composition.

In this work we tested inexpensive compounds for culture media elaboration, as sugarcane molasses, corn steep liquor and food grade sucrose. Sugarcane molasses is one of the by-products of the refinery process (18) that is traditionally used for culture media in various industrial fermentations (12). Molasses is composed of sugars (86°Bx), nitrogen compounds including crude proteins (8.2%), ash (16%) and fatty acids (0.3 - 1%) (U.S Sugar Corporation Molasses and Liquid Feeds Department - www.suga-lik.com/molasses/composition.html). Corn steep liquor is a by-product of the corn wet milling industry and it is composed of water (45 - 55%) free reducing sugars (0.1 - 11%), lactic acid (5 - 15%), ash (9 - 10%) and different nitrogen

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sources including ammonia and amino acids (2.7 - 4.5%) (7,14). Peptones are defined as protein hydrolysates that are soluble in water and not heat coagulable. They are obtained from plants, dairy proteins as casein or whey, and slaughterhouse waste and are widely used as nitrogen source for bacterial, yeast and fungal growth (13). Peptone is about 10% nitrogen and a less expensive amino acid source than individual amino acids. Some authors have shown the importance of amino acids in the enhancement of fruit aroma production by bacteria and filamentous fungi. Meza *et al.* (19) have screened some amino acids (arginine, histidine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine) for their ability to stimulate aroma production by *Ceratocystis fimbriata*. Spinnler and Djian (22) examined the effects of 20 amino acids on the aroma production by *Erwinia carotovora* subsp. *atroseptica*. In both studies leucine and valine, amino acids derived from pyruvate, presented the best results. In this work we have shown that molasses was better than food grade sucrose as a carbon source, when combined with peptone or corn steep liquor as a nitrogen source, allowing aroma production by *G. candidum* in an inexpensive culture media. Supplementation with the amino acids leucine, valine and alanine also increased fruity aroma production.

Media for testing production of aroma were prepared in 100 mL volumes in 200 mL Erlenmeyer flasks. The basic culture media were prepared using food grade sucrose (20 g/l) or Brazilian sugarcane molasses diluted to about 20 g sucrose /l (2.5 g in 100 mL of distilled water) as carbon sources; 4 g meat peptone/l or 1% v/v of Brazilian corn steep liquor as nitrogen sources. The amino acids added at 10 and 15 mM were valine, leucine, alanine, histidine, tyrosine, tryptophan, phenylalanine or methionine. Experiments were performed in duplicate by preparing a second batch of culture medium and a second inoculum. The inoculum of *G. candidum* (ATCC 1205) was prepared from one colony grown in a 500 mL Erlenmeyer flask containing 200 mL of 10 g meat peptone /l and 20 g glucose /l on a shaker at 250 r.p.m, for 3 days at 25±3°C, and 5 mL was used for each aroma production culture.

Culture aroma was monitored by sensory evaluation using a non-trained panel composed by three members with no restriction on descriptive terms used for the aroma quality (17,19). Aroma intensity was noted as: none (-), weak (+), medium (++) or strong (+++). Before each evaluation, a comparison was made with an uninoculated control flask and the evaluation was performed once a day, during four days.

The extraction of volatiles and further chemical analyses were done using *G. candidum* cultures grown in different culture media as described in Table 2. Volatiles were extracted from the headspace by Solid-Phase Micro Extraction (SPME) using 100 µm polydimethylsiloxane (Supelco, nº. 57300 - U) fibers. The extraction of volatiles was done in duplicate, by maintaining the fibers in contact with the headspace of the

culture flasks during 30 min, at 40°C and 60 r.p.m. After each extraction procedure, the fiber was conditioned at 250°C during 15 min. Volatile compounds were analysed using a gas chromatograph (Model: Agilent Technologies 6890N Network GC System™) equipped with a mass spectrometer (Model: Agilent 5973 Network™) and a nonpolar capillary column (HP5-MS; 30 m by 0.25 mm; 0.25-µm film thickness) at a helium flow rate of 8 psi. The oven temperature was held at 35°C for 5 min and then programmed to rise from 35 to 240°C at 7°C/min. This final temperature was maintained for 5 minutes. Data analysis was performed by comparing the mass spectrum of the compounds with the Wiley 275 L mass spectral data base and NIST MS Search Program (2002). In the case of the main esters (ethyl acetate, ethyl propionate, ethyl isovalerate, ethyl butyrate, and isoamyl isovalerate) were additionally confirmed by coincidence of each relative retention time with that of authentic standards.

G. candidum produced a fruity aroma when cultivated in media containing peptone or corn steep liquor with sugarcane molasses, however aroma was not detected by sensory evaluation when food grade sucrose was substituted as a carbon source. Sugarcane molasses based media containing peptone showed stronger aroma production in sensory evaluation than did media containing corn steep liquor. This should have been due to a higher amount of amino acids including 70% higher valine and 40% higher leucine in peptone that could have induced aroma compound production (5,10). The media containing corn steep liquor had a more alcoholic aroma especially after 72 h incubation. That may have been a result of the higher concentration of alanine making up more than 25% of the total nitrogen in corn steep liquor since degradation of this amino acid generates ethanol (7).

Supplementation of molasses - peptone or molasses - corn steep liquor media with some amino acids increased the aroma production by *G. candidum* as detected by sensory evaluation (Table 1). No aroma was produced with supplementation of amino acids when food grade sucrose was used rather than molasses. This may be because sugarcane molasses is more complex including different sugars, trace elements, vitamins and proteins (U.S. Sugar Corporation Molasses and Liquid Feed Department). Sugar cane molasses can have up to 9% of protein including 13% alanine, 6.6% valine, and 2.2% leucine that can stimulate volatile compounds production (18). Also according to Mee *et al.* (18) fatty acids including linoleic, palmitic and oleic acids are present up to 1%. Bañó *et al.* (2) have shown that long chain fatty acids induce lipase production by *G. candidum* in aqueous solutions thus enhancing ester synthesis. The best and most intense fruity aroma production by *G. candidum* resulted from supplementation of molasses and peptone medium with leucine, valine or alanine (Table 1). We found that supplementation with other amino acids yielded less intense or less desirable aromas. Similar results were reported

Table 1. Aroma intensity by sensory evaluation detected from *G. candidum* grown in molasses and peptone or corn steep liquor media supplemented with different amino acids.

Amino acid level	Nitrogen source	Leu ¹	Val	Ala	His	Trp	Tyr	Met	Phe
10mM	Peptone	++ ² Fruity	++ Fruity	++ Fruity and alcoholic	+ Fruity	+ Fruity	+ Fruity	++ Bad	++ Bad
15mM	Peptone	+++ Fruity	+++ Fruity	+++ Fruity and alcoholic	+ Fruity	+ Fruity	+ Fruity	++ Bad	+++ Bad
10mM	Corn steep Liquor	++ Fruity	++ Fruity	++ Fruity and alcoholic	+ +Fruity	+++ Bad	+++ Bad	ND ³	ND
15mM	Corn steep Liquor	++ Fruity	++ Fruity	++ Fruity and alcoholic	+ +Fruity	+++ Bad	+++ Bad	ND	ND

1. Leu = leucine; Val = valine; Ala = alanine; His = histidine; Trp = tryptophan; Phe = phenylalanine; Tyr = tyrosine; Met = methionine; None = control without amino acid addition; 2. Intensity = Weak (+); Medium (++); Strong (+++); 3. ND = no data.

· Without added amino acids the aroma intensity was weak (+) for both peptone and corn steep liquor media;

· With both Leucine and Valine added at 15 mM in Molasses peptone medium a strong (+++) fruity aroma was produced.

for the fungus *C. fimbriata* (19) and for the bacterium *Erwinia carotovora* subsp. *artroseptica* (22).

According to Yvon & Rijnen (25), different cheese microorganisms, including *G. candidum* are able to perform a multi-step pathway transforming amino acids to aroma compounds, including esters from the union of carboxylic acids with alcohols. Lipases may be responsible for this ester formation. For example, when leucine is used, 3-methylbutanol and 3-methylbutanoic acid (isovaleric acid) are formed and these compounds form esters such as isoamyl isovalerate and ethyl isovalerate. When valine is used, 2-methylbutanol and 2-methylbutanoic acid can be precursors of isobutyl isobutirate and ethyl isobutirate. These esters have been described as having a strong fruity aroma (9,15,20). When alanine was used, ethanol and acetic acid are formed (25) and these compounds can originate esters as ethyl acetate addition to the alcoholic aroma from the ethanol. Histidine addition resulted in the same weak fruit aroma in media without addition of extra amino acid, probably produced from the amino acids present on corn steep liquor, molasses and peptone. The compounds derived from histidine, apparently did not influence the aroma production. Methionine and the aromatic amino acids tryptophan, tyrosine and phenylalanine did not stimulate aroma production and, in some culture media, produced unpleasant odours (Table 1). This is probably explained by their chemical structure: methionine is a precursor of methanethiol and aromatic amino acids generate aromatic esters, compounds that do not have a desirable aroma.

The differences perceived in the aroma quality and intensity should reflect differences in the aroma compounds formed from different culture media. The aroma compounds produced by *G. candidum* from amino acid supplemented and non-

supplemented media are presented in Table 2. Ethanol, ethyl acetate and ethyl isovalerate were produced in both media without amino acid supplementation. Additional compounds detected in the peptone medium were ethyl isobutyrate, ethyl butyrate and ethyl tiglate; whereas in the corn steep liquor medium isoamyl alcohol, isobutyl isovalerate and isoamyl isovalerate were produced. Supplementation with individual aminoacids stimulated production of some additional aroma compounds. However, some of the volatiles produced in non-supplemented media were not detected with some of the amino acid supplements in the same media (Table 2).

The main volatile compounds we found in the headspace after cultivation of *G. candidum* were ethanol, ethyl acetate, ethyl isobutyrate, ethyl isovalerate, ethyl tiglate, isobutyl isovalerate and isoamyl isovalerate. These esters were classified by Cristiani & Monnet (8), Liu *et al.* (15) and Verstrepen *et al.* (23) as having strong fruity notes and the other esters we detected have similar sensory properties. The addition of 10 or 15 mM amino acid gave different results for production of some aroma compounds. The higher amino acid concentration yielded a positive result only about half of the time although duplicate results with the same amino acid concentration were consistent. We have confirmed the production of ester, alcohol and acid like fruit aroma as reported for *Geotrichum* by Daigle *et al.* (9), Damasceno *et al.* (10) and Pastore *et al.* (20).

Addition of the amino acids leucine, valine or alanine to culture media strongly enhanced *G. candidum* fruity aroma production, but alanine also resulted in an alcoholic aroma. Other inexpensive nutrient sources with high amounts of these amino acids could be tested. *G. candidum* produced mostly similar volatile compounds from the molasses with either

Table 2. The presence in the headspace of fruity volatile compounds produced by *G. candidum* from molasses medium with either peptone or corn steep liquor supplemented with different amino acids.

Volatile compound	Molasses + Peptone				Molasses + Corn Steep Liquor			
	None	Leu ¹	Val	Ala	None	Leu ¹	Val	Ala
Ethanol	28.3	3.3	24.7	24.9	1.6	2.8	5.7	12.7
Ethyl acetate	5.8	2.0	3.9	-	3.3	-	3.3	11.7
Isoamyl alcohol	-	-	-	-	10.9	-	-	3.2
Ethyl propionate	-	-	2.5	-	-	-	3.8	3.5
Ethyl isobutyrate	5.2	3.7	8.8	8.1	-	-	17.5	-
Ethyl butyrate	2.2	-	2.0	-	-	-	-	3.1
Ethyl isovalerate	50.4	54.3	41.8	48.4	55.0	63.4	47.9	44.9
Isobutyl isobutyrate	-	-	-	1.5	-	-	3.5	-
Ethyl tiglate	1.4	4.9	7.3	1.5	-	5.1	2.1	10.8
Propyl isovalerate	-	1.7	-	1.9	-	1.9	2.1	-
Isobutyl isovalerate	-	1.8	5.8	5.4	2.2	1.7	9.3	1.9
Isoamyl isovalerate	-	10.0	3.3	3.8	7.8	16.5	3.5	3.6
Isoamyl isobutyrate	-	7.4	-	-	-	-	-	-

1. Leu = leucine; Val = valine; Ala = alanine; His = histidine; None = control without amino acid addition; 2. Relative area of volatile compound (%).

peptone or corn steep liquor culture media that we tested. These media had similar amino acids compositions, including leucine, valine, alanine and histidine but at different concentrations. Distillers yeast and brewers yeast are readily available in regions with large fuel ethanol and beverage fermentation industries and have high levels of these amino acids (21). Micronized soybean containing 3.2% leucine, 2.2% valine and 1.8% alanine can be another possibility (1). Many of the esters produced by *G. candidum* could be used in the food industry as natural additives to improve the sensorial quality of products. We have shown that molasses, but not food grade sucrose, as a carbon source with peptone or corn steep liquor as a nitrogen source worked as inexpensive culture media for aroma production by *G. candidum*, and that supplementation with the amino acids leucine, valine and alanine increased this aroma production.

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RESUMO

Influência da composição do meio de cultivo e da suplementação com aminoácidos na produção de aroma frutal por *Geotrichum candidum*

Geotrichum candidum foi cultivado em diversos meios de cultura contendo sacarose ou melaço e milhocina ou peptona e a produção de aroma frutal foi verificada através de avaliação sensorial e cromatografia gasosa acoplada a espectrometria de massas. Os meios contendo melaço, peptona e leucina, valina ou alanina apresentaram os melhores resultados e a presença de diversos ésteres foi consistente com a formação de aroma frutal.

Palavras chave: *Geotrichum candidum*, aroma, milhocina, melaço, aminoácidos

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