MARIANA DIAS

APROVEITAMENTO DE RESÍDUOS DO PROCESSAMENTO DE CAFÉ PARA PRODUÇÃO DE CAROTENOIDES POR LEVEDURAS E BACTÉRIAS

LAVRAS – MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós Graduação em Microbiologia Agrícola, área de Biotecnologia de microrganismos aplicada a agropecuária e ao meio ambiente, para a obtenção do título de Doutor.

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APROVADA em 16 de dezembro de 2015.

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LAVRAS – MG

2015

Aos meus queridos pais, Eriberto e Iêda;
aos meus irmãos, Ana e Gabriel e
Ao meu esposo, Igor,
pelo incentivo e amor incondicional

DEDICO

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RESUMO

O café é um dos produtos agrícolas de maior importância no comércio mundial. Durante o seu processamento são geradas grandes quantidades de resíduos, cuja deposição no meio ambiente causa sérios problemas ambientais. A casca e a polpa do café são os principais subprodutos e apenas uma pequena parte é reaproveitada. Uma das alternativas é sua utilização como fonte de carbono em processos fermentativos, para a obtenção de produtos com valor agregado, como pigmentos microbianos. Os carotenoides compõem um grupo de pigmentos naturais amplamente distribuídos na natureza, com grande diversidade de estruturas e funções. São responsáveis pelas cores amarela, laranja e vermelha em uma variedade de plantas, animais e microrganismos. O trabalho foi realizado com os objetivos de selecionar um microrganismo capaz de utilizar os extratos de polpa e casca de café como fonte de carbono para a produção de carotenoides, determinar o melhor método de recuperação e extração desses pigmentos intracelulares e otimizar a produção. Além disso, buscou-se, ainda, avaliar o potencial antioxidante e antimicrobiano, e quantificar o teor de β-caroteno dos carotenoides obtidos. *Rhodotorula mucilaginosa* CCMA 0156 foi a cepa selecionada. Os três métodos de extração testados (acetona:metanol (7:3 v/v), acetona:éter de petróleo (1:1 v/v) e acetato de etila:éter de petróleo (1:1 v/v)) foram eficientes na recuperação dos carotenoides. A otimização da produção nos extratos de polpa e casca de café foi realizada pela metodologia de superfície de resposta. A máxima produção de carotenoides específicos (361,29±36,0 μg g^{-1}) foi obtida em meio com extrato de polpa (6,68%), glicose (2 g l^{-1}), peptona (10,04 g l^{-1}) e extrato de levedura (3 g l^{-1}). Os carotenoides específicos foram 296,58±13,2 em meio com extrato de casca (8,36%) contendo glicose (6,36 g l^{-1}) e peptona (3,68 g l^{-1}). Os carotenoides produzidos apresentaram atividades antioxidante e antimicrobiana contra bactérias patogênicas, *Salmonella cholerasus*, *Escherichia coli*, *Staphylococcus aureus* e *Listeria monocytogenes* e fungos toxigênicos, *Aspergillus flavus*, *A. parasiticus*, *A. carbonarius* e *A. ochaceus*. β-caroteno representou de 13% a 20% da produção de carotenoides totais em extrato de polpa e extrato de casca de café, respectivamente.

Palavras-chave: Polpa de café. Casca de café. β-caroteno. *Rhodotorula mucilaginosa*. Fermentação submersa. Metodologia de superfície de resposta.

ABSTRACT

Coffee is one of the most important agricultural products in world trade. During its processing a large amount of residues are created, the deposition of which causes serious environmental problems. The bark and coffee pulp are the main by-products but only a small part of it can be reused. One alternative is to use them as a carbon source in fermentation processes for obtaining value-added products, such as microbial pigments. Carotenoids comprise a group of natural pigments widely distributed in nature with wide variety of structures and functions. They are responsible for yellow, orange and red in a variety of plants, animals and microorganisms. The study aimed to select a microorganism able to use the pulp and coffee husk extract as a carbon source for the production of carotenoids, determine the best method of recovery and extraction of these intracellular pigments and optimize production. Moreover, the study tries to evaluate the antioxidant and antimicrobial potential. Finally, to assess the β-carotene content present in the carotenoids extracts. *Rhodotorula mucilaginosa* CCMA 0156 strain was selected. The three tested extraction methods (acetone: methanol (7: 3 v / v) acetone: petroleum ether (1: 1 v / v) ethyl acetate: petroleum ether (1: 1 v / v)) were efficient in the recovery of carotenoids. Optimization of carotenoid production was achieved by experimental design technique. Maximum concentration of specific carotenoids (361.29±36.0 μg g^{-1}) was obtained in pulp extract (6.68 %) medium containing 2 g l^{-1} glucose, 10.04 g l^{-1} peptone, 3 g l^{-1} yeast extract. Specific carotenoids (296.58±13.2) in husk extract (8.36 %) medium containing 6.36 g l^{-1} glucose and 3.68 g l^{-1} peptone. Carotenoids produced exhibited antioxidant and antimicrobial activities against pathogenic bacteria: *Salmonella cholerasus*, *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes* and toxigenic fungi: *Aspergillus flavus*, *A. parasiticus*, *A. carbonarius* and *A. ochaceus*. β-carotene represented 13 and 20% of the total carotenoids produced in coffee pulp extract and husk extract, respectively.

Key words: Coffee pulp. Coffee husk. β-carotene. *Rhodotorula mucilaginosa*. Submerged fermentation. Response surface methodology.

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PRIMEIRA PARTE

1 INTRODUÇÃO

A cor é um atributo importante que determina a aceitação dos alimentos pelo consumidor. A adição de agentes de cor em alimentos processados tem sido uma prática comum há muitos anos. Devido à possível toxicidade dos corantes artificiais, corantes naturais alternativos têm sido cada vez mais procurados (ZENI et al., 2011).

Os carotenoides compõem um grupo de pigmentos naturais amplamente distribuídos na natureza, com grande diversidade de estruturas e funções. São responsáveis pelas cores amarela, laranja e vermelha em uma variedade de plantas, animais e microrganismos (BOTELLA-PAVÍA; RODRÍGUEZ-CONCEPCIÓN, 2006; CABRAL et al., 2011).

A demanda industrial por pigmentos carotenoides, tais como β-caroteno e astaxantina, tem aumentado devido à grande variedade de aplicações como corantes nas indústrias de alimentos, farmacêutica, cosmética e de rações (AKSU; EREN, 2005; FRENGOVA; BESHKOVA, 2009).

Mais de 90% do mercado mundial dos carotenoides corresponde à síntese química, mas a demanda de crescimento de aditivos naturais faz da produção microbiológica um mercado promissor (VILA; COUSO; LEO'N, 2008). Nos últimos anos, cresceu o interesse pela produção de carotenoides naturais por meio de fermentação microbiana (DAS et al., 2007). Vários microrganismos, incluindo bactérias, fungos, leveduras e algas, são capazes de sintetizar carotenoides (FRENGOVA; BESHKOVA, 2009). A produção microbiana de carotenoides vem se destacando devido a fatores tais como possibilidade de utilização de substratos de baixo custo, denominação de substâncias naturais, pequeno espaço para produção, não estando sujeita às

condições ambientais como clima, estação do ano ou composição do solo, e controle das condições de cultivo (AKSU; EREN, 2005; VALDUGA et al., 2009a).

O café é uma das bebidas mais populares do mundo e tem crescido constantemente, em importância comercial, durante os últimos 150 anos (DAGLIA et al., 2000). O café é a segunda maior *commodity* negociada no mundo e gera grande quantidade de resíduos/subprodutos durante o processamento, aproximadamente 8,6 milhões de toneladas (INTERNATIONAL COFFEE ORGANIZATION - ICO, 2013; MUSSATTO et al., 2011; NABAIS et al., 2008). Independente do método de processamento empregado, ou seja, processo via úmida, semisseca ou seca, resíduos sólidos de torrefação e fermentação, como polpa e casca, são obtidos. As cascas e a polpa de café compõem cerca de 45% do fruto e são os principais subprodutos da agroindústria cafeeira (ESQUIVEL; JIMÉNEZ, 2012).

A polpa e a casca de café são materiais potenciais para serem utilizados como substratos em bioprocessos (BRAND et al., 2000) como fonte de carbono para a obtenção de produtos químicos e fermentados com valor agregado, como enzimas, alcoóis, proteínas, ácidos orgânicos, aminoácidos, metabólitos secundários biologicamente ativos e compostos de aroma que são de grande interesse para as indústrias farmacêutica, de cosméticos e de alimentos (LAUFENBERG; KUNZ; NYSTROEM, 2003; MURTHY et al., 2012; WYMAN, 2003).

Diante do exposto, no presente estudo buscou-se alcançar os seguintes objetivos:

a) selecionar bactérias e leveduras capazes de crescer em meios de cultivo com extrato de casca e extrato de polpa de café como únicas fontes de carbono;

b) produzir carotenoides de leveduras e bactérias em meios de cultivo contendo extrato de casca e extrato de polpa de café como únicas fontes de carbono;
c) avaliar diferentes combinações de solventes orgânicos para a extração e a recuperação dos carotenoides intracelulares;
d) otimizar a produção de carotenoides em meios contendo extrato de casca e extrato de polpa de café pela levedura *Rhodotorula mucilaginosa* CCMA 0156;
e) avaliar o potencial antimicrobiano e antioxidante dos carotenoides produzidos;
f) quantificar o teor de β-caroteno presente nos carotenoides totais por cromatografia líquida de alta eficiência.

2 REFERENCIAL TEÓRICO

2.1 Pigmento

A palavra “pigmento” tem origem latina e, originalmente, denominava uma cor (no sentido de matéria corante). Mais tarde, foi estendida para indicar adereços coloridos (maquiagem, por exemplo). No início da Idade Média, a palavra foi utilizada também para descrever os mais diversos tipos de extratos de plantas e vegetais, especialmente aqueles utilizados para a coloração de alimentos. A palavra pigmento ainda vem sendo empregada nesse sentido na terminologia biológica: matéria corante presente em animais ou plantas e que ocorre em grânulos dentro das células ou membranas celulares, como depósitos nos tecidos, ou suspensa nos fluidos corpóreos (ULLMANN’S..., 1985).

O significado moderno associado à palavra “pigmento” (em inglês, *pigment*) foi atribuído no século XX, significando uma substância constituída de pequenas partículas que é praticamente *insolúvel* no meio aplicado e é utilizada devido às suas propriedades corantes, protetivas ou magnéticas (ULLMANN’S..., 1985). Essa definição aplica-se bem aos pigmentos de origem mineral, como dióxido de titânio ou negro de fumo; para materiais corantes solúveis, geralmente compostos orgânicos, é mais adequado utilizar a expressão “corante” (em inglês, *dye*). No entanto, ambos os termos (corante e pigmento) são empregados para denominar substâncias utilizadas para conferir cor a alimentos, às vezes indistintamente (ABEROUMAND, 2011; ALEXANDRA et al., 2001; BEYER et al., 2002).

2.1.1 Pigmentos naturais x sintéticos

Muitos dos alimentos consumidos atualmente contêm aditivos, sendo a maioria de origem sintética. Estes aditivos podem ser corantes, antioxidantes, conservantes, emulsificantes, estabilizadores e outros aditivos, como realçadores de sabor e agentes antiespumantes (ABEROUMAND, 2011). Os alimentos tradicionalmente coloridos de forma artificial no Brasil são as balas e os pós para refresco, além de cereais, iogurtes, sorvetes, bebidas, produtos cárneos, lácteos e ração (MANCZYK, 2007).

De acordo com Brasil (2008), aditivos para alimentos são definidos como "substâncias ou misturas de substâncias, dotadas ou não de poder alimentício, adicionadas aos alimentos com a finalidade de lhes conferir ou intensificar o aroma, a cor, o sabor ou modificar seu aspecto físico geral, ou ainda prevenir alterações indesejáveis". Excluem-se ainda da definição posterior os sucos e/ou os extratos de vegetais e outros ingredientes utilizados na elaboração de alimentos (e bebidas) que têm coloração própria, salvo se adicionados com a finalidade de conferir ou intensificar a coloração própria do produto.

Nesse sentido, corantes são aditivos alimentares definidos como "toda substância ou a mistura de substâncias que possuem a propriedade de conferir, intensificar ou restaurar a cor de um alimento e/ou bebida" (BRASIL, 2008, p. 18). Conforme Brasil (2008), considera-se corante orgânico natural aquele obtido a partir de vegetal ou, eventualmente, de animal, cujo princípio corante tenha sido isolado com o emprego de processo tecnológico adequado. O corante orgânico sintético é obtido por síntese orgânica mediante o emprego de processo tecnológico adequado. Já o corante artificial é a substância obtida por processo de síntese (com composição química definida).

A cor é um importante atributo que determina a aceitação dos alimentos pelos consumidores (ABEROUMAND, 2011). A adição de corantes em alimentos processados tem sido uma prática comum há muitos anos. Devido à possibilidade de toxicidade dos corantes artificiais, alternativas naturais de corantes têm sido cada vez mais procuradas (ZENI et al., 2011). A utilização de pigmentos naturais em alimentos tem aumentado nos últimos anos, devido às vantagens do *marketing* empregando ingredientes naturais aos produtos e a preocupação dos consumidores com relação a eventuais efeitos nocivos de pigmentos sintéticos (HU et al., 2012).

A substituição dos pigmentos sintéticos utilizados em alimentos por pigmentos naturais será um importante passo para produzir alimentos mais saudáveis, pois, além de fornecer a cor, muitos têm propriedades funcionais e/ou terapêuticas (DERNER, 2006).

Os maiores obstáculos para a exploração dos novos corantes derivados de fontes naturais são a legislação atual, que requer testes toxicológicos caros; o custo de processamento (incluindo o cultivo ou produção biotecnológica) e a aceitação pelos consumidores (de um material previamente não conhecido) (BOTELLA-PAVÍA; RODRÍGUEZ-CONCEPCIÓN, 2006).

A notoriedade que os corantes naturais vêm assumindo deve-se não só à tendência mundial de consumo de produtos naturais, mas também às propriedades funcionais atribuídas a alguns desses pigmentos. O mercado estimula cada vez mais o desenvolvimento de novos estudos, no intuito de superar as limitações tecnológicas existentes (MATA-GÓMEZ et al., 2014).

2.1.2 Pigmentos microbianos

A maioria dos precursores dos corantes químicos é, geralmente, derivada de produtos petroquímicos, um fato que aumenta a preocupação de

consumidores quanto à segurança da ingestão, por tempo prolongado, dessas substâncias (VALDUGA et al., 2009c). É conhecido que o aumento da restrição dessas substâncias no futuro pode eliminar alguns dos corantes sintéticos aprovados. Consequentemente, é necessário encontrar fontes alternativas de corantes de alimentos. Um método alternativo é a produção desses corantespor meio de processos microbianos (DUFOSSÉ, 2006; FRENGOVA; BESHKOVA, 2009).

Há um crescente interesse, na área da biotecnologia, pela obtenção de fontes não vegetais de corantes. Dessa forma, pigmentos naturais de origem microbiana são uma alternativa promissora em relação a outros aditivos extraídos de animais ou vegetais (CARVALHO et al., 2006). A produção de pigmentos microbianos apresenta vantagens, como cultivo contínuo e rápido crescimento, alta produtividade, utilização de resíduos agroindustriais baratos e otimização da produção, além da grande variedade de microrganismos produtores e a diversidade de pigmentos encontrados (DUFOSSÉ et al., 2005; DURÁN et al., 2012; RANGEL-YAGUI et al., 2004).

A natureza é rica em cores e microrganismos produtores de pigmentos (fungos, leveduras, bactérias, cianobactérias e microalgas). Entre as moléculas produzidas por microrganismos estão carotenoides, melaninas, flavinas, quinonas, monascinas, violaceínas, ficocianinas, prodigiosinas e derivados de fenazina (DUFOSSÉ, 2006; DUFOSSÉ et al., 2005; DURÁN et al., 2012).

Há muitos estudos sobre as prodigiosinas e suas atividades biológicas como antibacterianas, antifúngicas, imunossupressivas e anticancerígenas (MONTANER; PÉREZ-TOMÁS, 2003; WILLIAMSON et al., 2007). Além de espécies de *Serratia*, muitas espécies de bactérias pertencentes aos gêneros *Streptomyces Actinomadura*, *Pseudomonas*, *Pseudoalteromonas* e outras, como *Hahella*, *Vibrio* e *Zooshikella*, têm sido relatadas como produtoras do pigmento

vermelho (ABEROUMAND, 2011; SOLIEV; HOSOKAWA; ENOMOTO, 2011).

Em relação aos carotenoides, bactérias dos gêneros *Agrobacterium*, *Paracoccus*, *Streptomyces*, *Mycobacterium*, *Rhodococcus* e *Deinococcus* têm sido relatadas como produtoras desses pigmentos (LEE et al., 2004; SOLIEV; HOSOKAWA; ENOMOTO, 2011; TIAN; HUA, 2010; VALDUGA et al., 2009c). O pigmento violeta violaceína é um derivado de indol, predominantemente isolado de bactéria do gênero *Chromobacterium*, que habita o solo e a água de áreas tropicais e subtropicais (DURÁN et al., 2012; RETTORI; DURÁN, 1998). As fenazinas são compostos aromáticos produzidos por uma grande variedade de gêneros bacterianos, incluindo *Streptomyces*, *Pseudomonas*, *Pelagibacter* e *Vibrio* e actinobactérias, como *Actinomycetes* (PIERSON; PIERSON, 2010).

Há uma crescente evidência científica de que os pigmentos microbianos podem ter benefícios potenciais na saúde humana e animal, como atividades anticarcinogênicas, imunomoduladoras e antioxidantes, o que tem aumentado o interesse comercial pela busca de fontes alternativas naturais (GHARIBZAHEDI; RAZAVI; MOUSAVI, 2013; RIBEIRO; BARRETO; COELHO, 2011; SOLIEV; HOSOKAWA; ENOMOTO, 2011).

2.2 Carotenoides

Os carotenoides pertencem a um grupo de mais de 600 moléculas que podem ser encontradas em diferentes formas de vida e com diversas funções, que vão desde o seu papel original evolutivo, como a fotossíntese, como pigmentos antioxidantes até precursores da vitamina A (FRENGOVA; BESHKOVA, 2009; WALTER; STRACK, 2011).

Os carotenoides apresentam um grupo importante de pigmentos naturais com aplicações específicas como corantes, suplementos alimentares e nutracêuticos, medicamentos e cosméticos. O interesse por carotenoides tem aumentado nos últimos anos, devido à sua comprovada atividade pró-vitamínica A e pela evidência de outras propriedades biológicas, tais como atividades imunomoduladoras, anticarcinogênicas, prevenção de degeneração macular e de doenças cardiovasculares (KRINSKY; JOHNSON, 2005). Além disso, o mercado de corantes naturais tem se mostrado muito promissor, devido à tendência de se evitar alimentos que contenham aditivos artificiais (KAISER et al., 2007; SQUINA; MERCADANTE, 2003).

Os carotenoides têm sido amplamente utilizados como corantes nas indústrias de alimentos, farmacêutica, cosmética e de rações (SQUINA; MERCADANTE, 2003; VALDUGA et al., 2009c). Industrialmente, os carotenoides, tais como β-caroteno e astaxantina, são utilizados como corantes naturais para alimentos ou adicionados à ração para aquicultura (AKSU; EREN, 2007; GHARIBZAHEDI; RAZAVI; MOUSAVI, 2013).

O enorme mercado internacional de carotenoides foi atendido, principalmente, por carotenoides sintéticos com estruturas idênticas àquelas de carotenoides naturais. Tradicionalmente, os carotenoides têm sido comercializados como um pó seco ou a partir de extratos de plantas, tais como urucum, páprica e açafrão (ZENI et al., 2011). A desvantagem dos corantes naturais obtidos de plantas é que eles sofrem uma diminuição ou instabilidade da matéria-prima, sujeita às condições climáticas, bem como pela variação do nível de coloração e qualidade do produto final (MALDONADE; RODRIGUEZ-AMAYA; SCAMPARINI, 2012).

2.2.1 Estrutura e biossíntese

Os carotenoides são um subgrupo de compostos isoprenoides lipofílicos compreendendo mais de 700 estruturas (BRITTON, 2008). A grande maioria dos carotenoides é derivada de tetraterpenoides (C_{40}) compostos de oito unidades isoprenoides, ligados de tal forma que a molécula é linear e simétrica, com a ordem invertida no centro. Entretanto, carotenoides C_{30} e C_{50} podem ser produzidos por certas bactérias via compostos intermediários diferentes (WALTER; STRACK, 2011).

Os carotenoides são tetraterpenos e sua biossíntese apresenta um padrão para todos os terpenoides. O primeiro precursor específico na biossíntese dos terpenoides é o ácido mevalônico. A estrutura básica acíclica C_{40} pode ser modificada por reações de hidrogenação, desidrogenação, ciclização ou oxidação (Figura 1). Os carotenoides hidrocarbonetos são chamados de carotenos e os derivados oxigenados, de xantofilas. A característica de absorção de luz destes pigmentos dá-se devido à cadeia de duplas ligações conjugadas que atua como cromóforo, sendo necessárias, aproximadamente, sete ligações duplas conjugadas para que o carotenoide apresente coloração (VALDUGA et al., 2009c; WALTER; STRACK, 2011).

Figura 1 Fluxograma dos estágios da biossíntese de carotenoides
Fonte: Valduga et al. (2009c)

Algumas bactérias sintetizam carotenoides mais curtos C_{30} de precursores C_{15} farnesil difosfato ou produzem carotenoides de cadeia longa C_{45} e C_{50}. Exemplos desses carotenoides exóticos são estafiloxantina (C_{30}), identificada em *Staphylococcus aureus* e poucas outras espécies (PELZ et al., 2005), bem como decaprenoxantina (C_{50}) de *Corynebacterium glutanicum* (KRUBASIK; KOBAYASHI; SANDMANN, 2001), exibindo adições prenil no exterior de dois anéis de ciclo-hexeno terminais.

Duas classes de carotenoides são encontradas na natureza: i) os carotenos, tais como β-caroteno, hidrocarbonetos lineares que podem ser ciclizados em uma ou ambas as extremidades da molécula e ii) os derivados oxigenados de carotenos, como luteína, violaxantina, neoxantina e zeaxantina, denominados xantofilas (BOTELLA-PAVÍA; RODRÍGUEZ-CONCEPCIÓN, 2006). Na Figura 2 apresentam-se as estruturas de algumas xantofilas (zeaxantina, luteína, criptoxantina e astaxantina) e carotenos (neurosporeno, licopeno, β-caroteno e α-caroteno).

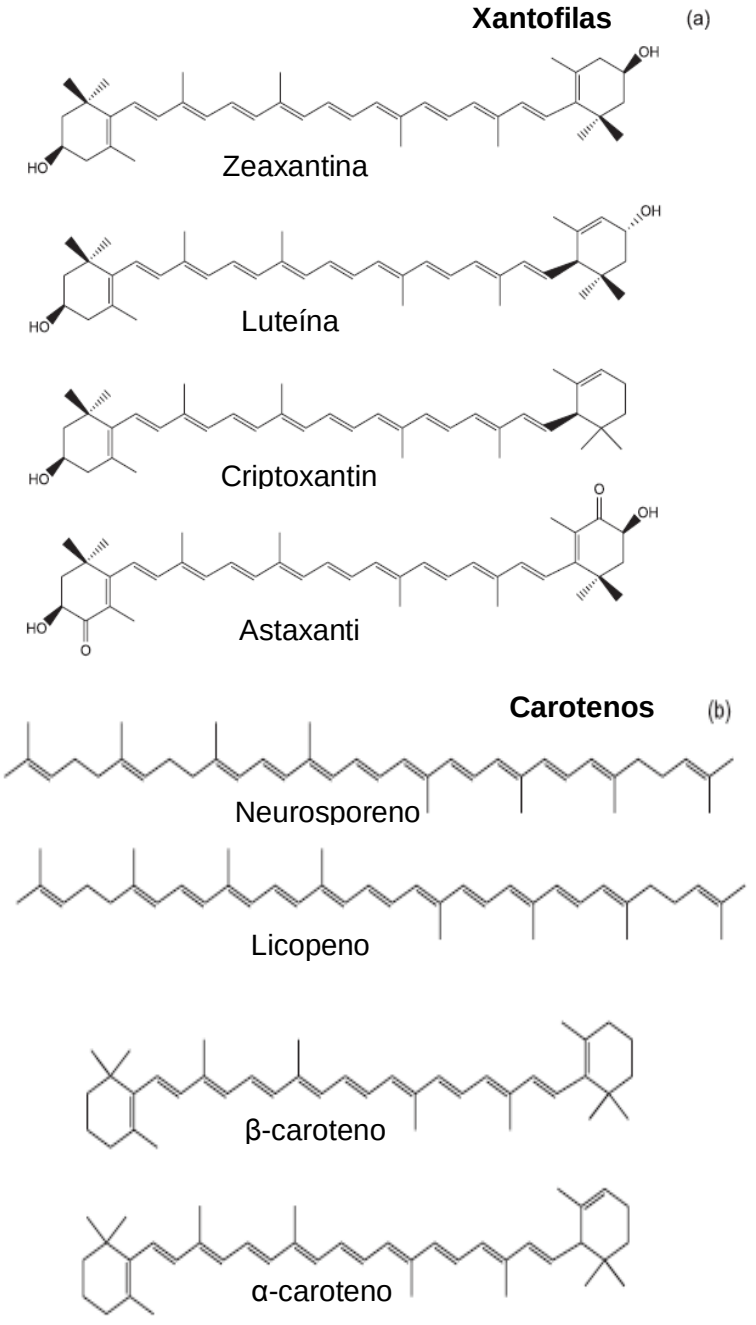

Figura 2 Estrutura química de alguns carotenoides a) xantofilas e b) carotenos
Fonte: Valduga et al. (2009c)

A via de biossíntese de carotenoides por microrganismos comumente envolve três etapas que são: (1) a conversão de acetil-CoA em 3-hidroxi-3-metil glutaril-CoA (HMG-CoA), que é catalizada pela enzima HMG-CoA sintase. HMG-CoA é, então, convertido a um composto de seis carbonos, ácido mevalônico (MVA), que é o primeiro precursor específico da via biossintética de terpenoides. O mevalonato (MVA) é, posteriormente, convertido em isopentenil pirofosfato (IPP) por uma série de reações que envolvem fosforilação pela mevalonato quinase, seguida por descarboxilação; (2) o isopentenil pirofosfato (IPP) é isomerizado a dimetilallil pirofosfato (DMAPP) com a adição sequencial de três moléculas de IPP a DMAPP. Estas reações são catalizadas pela prenil transferase para produzir o composto de 20 carbonos, o geranil geranilpirofosfato (GGPP). A condensação de duas moléculas de GGPP gera o fitoeno (carotenoide C_{40} da via), o qual sofre dessaturação para formar o licopeno; (3) como o licopeno é um composto *trans*, a isomerização da primeira e da segunda dupla-ligação do fitoeno deverá ocorrer durante a etapa de dessaturação (BHOSALE; BERNSTEIN, 2005; FRENGOVA; BESHKOVA, 2009; SCHMIDT et al., 2011). Na Figura 3 apresenta-se a biossíntese dos carotenoides por microrganismos.

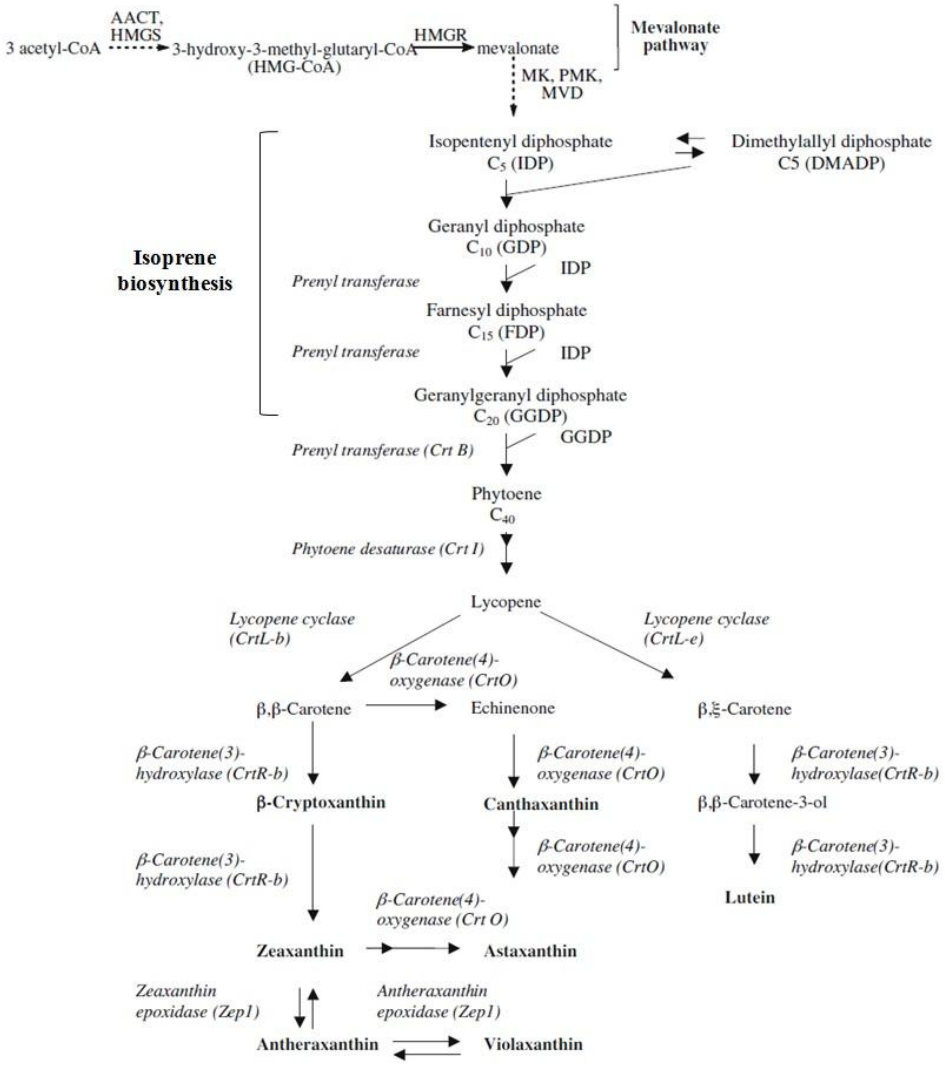

Figura 3 Possíveis vias biossintéticas para a formação de alguns carotenoides em bactérias, algas e leveduras

Fonte: Bhosale e Bernstein (2005) e Schmidt et al. (2011), modificado

2.2.2 Microrganismos produtores de carotenoides

Os carotenoides são moléculas de grande interesse científico por causa de suas propriedades únicas, ampla distribuição e diversas funções (BHOSALE,

2004). Em organismos fotossintéticos, as moléculas de carotenoides são utilizadas para absorver a luz. Em muitos organismos, o seu papel principal é o de atuar como um antioxidante ao neutralizar os radicais livres e, assim, prevenir o dano oxidativo potencial para as células (EL-AGAMEY et al., 2004; KRINSKY et al., 2003; LIANG et al., 2006). Liu et al. (2005) demonstraram que um mutante de *Staphylococcus aureus* com a biossíntese de carotenoides interrompida é mais susceptível à morte oxidativa, indicando que os carotenoides poderiam atuar como um fator de virulência. Os carotenoides podem reduzir a penetração de oxigênio singleto, diminuindo a fluidez da membrana (SUBCZYNSKI et al., 1991). Relatos recentes feitos por Kamila et al. (2008) mostraram que os carotenoides polares, tais como zeaxantina, podem mediar a transferência de prótons transmembrana *in vivo*.

Os carotenoides de fontes microbianas têm atraído muita atenção nos últimos anos. A principal razão para o interesse em utilizar microrganismos para produzirem compostos em relação aos isolados de plantas e animais ou sintetizados quimicamente é a facilidade de aumentar a produção microbiana pelas condições de cultivo e pela manipulação genética (MALDONADE; RODRIGUEZ-AMAYA; SCAMPARINI, 2008).

A utilização comercial dos microrganismos com potencial biotecnológico para a produção de carotenoides ainda é limitada pelo elevado custo de produção. No entanto, o custo de produção de carotenoides por fermentação pode ser minimizado pela utilização de subprodutos agroindustriais baratos, como fontes de nutrientes (AKSU; EREN, 2005). A produção de carotenoides a partir de diferentes substratos tem sido relatada em alguns trabalhos com grãos (aveia, trigo, cevada, milho, arroz, centeio), lipídios, glicerol, celobiose, cana-de-açúcar, melaço, mosto de uva, queijo e soro de leite, por diferentes cepas microbianas (AKSU; EREN, 2005; BHOSALE; GADRE, 2001; SCHNEIDER et al., 2013; VALDUGA et al., 2008, 2009b).

Os tipos de carotenoides e as quantidades produzidas podem variar dependendo do microrganismo, do meio de cultura e das condições operacionais (temperatura, pH, taxa de aeração e luminosidade). Muitos dos estudos são realizados objetivando a otimização das condições de cultura que diretamente afetam o crescimento dos microrganismos e a produção de carotenoides (VALDUGA et al., 2009c).

A possibilidade da produção de corantes naturais em escala industrial e o elevado valor dos produtos tornam a produção biotecnológica de carotenoides uma área de intenso estudo (VALDUGA et al., 2009c). A produtividade de um bioprocesso em um dado sistema depende das condições nutricionais e físicas da cultura, afetando não somente o crescimento celular como a produção de pigmento (LIU; WU, 2007). Sendo assim, os microrganismos acumulam vários tipos de carotenoides como resposta ao estresse das condições ambientais (BHOSALE, 2004).

Aksu e Eren (2005) avaliaram os efeitos de pH, temperatura, taxa de aeração, concentrações de açúcar inicial, e sulfato de amônio e adição de ativadores (óleo de semente de algodão e tween 80) sobre o crescimento e a produção de carotenoides pela levedura *Rhodotorula mucilaginosa.* O pH ótimo e a temperatura determinados na produção foram de 7,0 e 30 °C, respectivamente. A concentração inicial de 2 g/L de $(NH_4)_2SO_4$ e 20 g/L de melaço de cana forneceu a máxima produção de carotenoides totais (89,0 mg/L).

Em geral, os valores máximos de carotenoides totais não estão diretamente correlacionados aos valores máximos de produção de biomassa (BUZZINI et al., 2005). A máxima produção de carotenoides observada por Fang e Chiou (1993) ocorreu depois de 35 horas de fermentação, enquanto o crescimento celular máximo ocorreu após 25 horas. Johnson e Gil-Hwan (1991) verificaram que o pH ótimo de crescimento para *Phaffia rhodozyma* foi de 5,8, enquanto a máxima produção de astaxantina foi a pH 5,0.

A sacarose e a glicose são as formas de carbono mais comumente utilizadas na bioprodução de carotenoides. O uso de glicose pode induzir a maiores rendimentos na produção específica de carotenoides (1.000 $\mu g.g^{-1}$) por *Rhodotorula* sp. (BUZZINI; MARTINI, 2000). Porém, na produção de astaxantina por *P. rhodozyma*, Sutherland et al. (1996) verificaram que concentrações altas de glicose inibem a carotenogênese, enquanto sacarose, maltose e celobiose foram os dissacarídeos que produziram melhores resultados.

Chen, Han e Gu (2006) estudaram a otimização do meio fermentativo para a produção de carotenoides por *Rhodobacter sphaeroides* e concluíram que a adição de $MgSO_4$ 0,12 $g.L^{-1}$, Na_2HPO_4 2,05 $g.L^{-1}$, $FeSO_4$ 0,03 $g.L^{-1}$, Na_2CO_3 2,22 $g.L^{-1}$no meio de cultivo facilita a acumulação de carotenoides por essa levedura, cuja máxima produção foi 17,2 $mg.L^{-1}$.

Sporidiobolus salmonicolor em processo de otimização produziu 1,019 $\mu g\ L^{-1}$ de carotenoides totais em meio contendo 40 $g\ L^{-1}$ glicose, 10 $g\ L^{-1}$ extrato de malte a 14 $g\ L^{-1}$ peptona, a 180 rpm, 25 °C e pH inicial de 4,0 (VALDUGA et al., 2009b).

Leveduras dos gêneros *Rhodotorula* e *Sporobolomyces* foram cultivadas em caldo YM (*yest malt*), a 200 rpm, 25 °C, por 5 dias, sem iluminação. Os principais carotenoides obtidos foram toruleno e β-caroteno. *R. glutinis* foi a levedura que apresentou a maior produção de carotenoides totais (881 μg/L) (MALDONADE; RODRIGUEZ-AMAYA; SCAMPARINI, 2008).

A produção biotecnológica de carotenoides vem se destacando devido a fatores tais como possibilidade de utilização de substratos de baixo custo para a bioprodução; denominação de substâncias naturais; pequeno espaço para produção, não estando sujeita às condições ambientais como clima, estação do ano ou composição do solo, e controle das condições de cultivo (AKSU; EREN, 2005; VALDUGA et al., 2009a).

Na literatura há relatos de alguns gêneros de microrganismos produtores de carotenoides, como *Rhodotorula*, *Rhodosporidium*, *Sporobolomyces*, *Candida*, *Cryptococcus*, *Phaffia* (leveduras), *Blakeslea*, *Monascus*, *Penicillum* (fungos filamentosos), *Nannochloropsis salina* e *Dunaliella salina* (microalgas) (AKSU; EREN, 2005; CHEN; VAIDYANATHAN, 2013; FRENGOVA; BESHKOVA, 2009; HU et al., 2012; MALDONADE; RODRIGUEZ-AMAYA; SCAMPARINI, 2008). Além destes, outros microrganismos produtores de pigmentos incluem bactérias dos gêneros *Mycobacterium*, *Micrococcus*, *Staphylococcus*, *Flavobacterium*, *Rhodobacter*, *Rhodococcus* e *Streptomyces* (KAISER et al., 2007; MALDONADE, 2003; PELZ et al., 2005; VALDUGA et al., 2009c) (Tabela 1).

Tabela 1 Microrganismos produtores de carotenoides

Microrganismos	Principais carotenoides produzidos
Cianobactérias	
Anabaena variabilis	Castaxantina
Nostoc commune	Castaxantina
Algas	
Chlorela pyrenoidosa	Luteína
Dictycoccus cinnabarinus	Cantaxantina
Dunaliella salina	β-caroteno
Dunaliella tertiolecta	β- caroteno
Haematococcus pluvialis	Astaxantina
Spongiococcum excetricum	Luteína
Fungos e leveduras	
Rhodotorula glutinis	Toruleno
Blakeslea trispora	β- caroteno e licopeno
Rhodotorula mucilaginosa	Tolureno, torularrodina, β-caroteno
Rhodotorula rubra	β- caroteno
Phaffia rhodozyma	Astaxantina, β-caroteno, zeaxantina
Sporobolomyces roseus	β- caroteno, tolureno
Xanthophyllomyces dendrorhous	Astaxantina
Bactérias	
Mycobacterium	Cantaxantina, astaxantina
Rhodococcus	Cantaxantina
Flavobacterium sp.	Zeaxantina
Streptomyces	Xantofilas

Fonte: Valduga et al. (2009c), modificado; Kaiser et al. (2007), Maldonade (2003) e Pelz et al. (2005)

Lee et al. (2004) relataram uma nova espécie de *Paracoccus haeundaensis*, uma bactéria gram-negativa, halofílica, como produtora de astaxantina. Altos níveis de licopeno (2 mg. g^{-1} peso seco de células ou até 15 mg. L^{-1} de cultura) foram detectados em trabalho de Wang et al. (2012), por uma bactéria fotossintetizante, *Rhodospirillum rubrum.*

Muitos microrganismos produzem carotenoides, porém, nem todos os microrganismos são industrialmente interessantes. As leveduras destacam-se

pelo seu uso como fonte proteica, pela capacidade de crescimento em substratos de baixo custo e pelo alto teor de açúcar (VALDUGA et al., 2011).

A biossíntese de carotenoides em leveduras está envolvida com os mecanismos de resposta ao estresse (BHOSALE, 2004). Leveduras dos gêneros *Rhodotorula*, *Rhodosporidium* e *Phaffia* têm sido descritas como capazes de produzir carotenoides sob condições de cultivo desfavoráveis, incluindo radiação UV, temperatura, solventes e metais pesados (BREIEROVA et al., 2008; BUZZINI, 2001).

Os produtos que contêm carotenoides obtidos a partir de fontes microbianas, como *Dunaliella salina*, *Blakeslea trispora* (β-caroteno), *Haematococcus* sp., *Xanthophyllomyces dendrorhous* (astaxantina) e a levedura *Phaffia rhodozyma* (astaxantina, β-caroteno), estão atualmente disponíveis no mercado (KIM; SEO; PARK, 1997; RIBEIRO; BARRETO; COELHO, 2011; RODRÍGUEZ-SÁIZ; DE LA FUENTE; BARREDO, 2010). A síntese dos diferentes carotenoides por várias leveduras, como as do gênero *Rhodotorula*, tem levado a considerar estes microrganismos como potenciais fontes de pigmentos naturais (LIBKIND; BROOCK, 2006; SCHNEIDER et al., 2013).

2.2.3 Métodos de extração de carotenoides

Os carotenoides de leveduras e bactérias se acumulam em partículas lipídicas na célula e, dessa forma, podem ser extraídos utilizando-se diferentes solventes orgânicos (ex: acetona, éter de petróleo, hexano, éter etílico, diclorometano, metanol). Porém, ainda não há nenhuma técnica padrão que garanta o máximo rendimento da extração para qualquer microrganismo (VALDUGA et al., 2009a).

A extração, geralmente, requer um rompimento eficiente da membrana celular. Tratamentos ácidos podem ser utilizados na lise da membrana celular.

Porém, podem resultar em perda considerável dos carotenoides. Outros métodos de extração dos carotenoides intracelulares baseados em reações enzimáticas (FANG; WANG, 2002; SOREBAKKEN et al., 2004) e extração com dióxido de carbono supercrítico já foram relatados (LIM et al., 2002; MONTERO et al., 2005). Monks et al. (2012) avaliaram tanto métodos químicos quanto enzimáticos e ultrassom para rompimento celular e obtenção de carotenoides produzidos pela levedura *Sporidiobolus salmonicolor* (CBS 2636). A concentração máxima de carotenoides totais (2.875 g/L) foi obtida do tratamento com CO_2 supercrítico (300 bar/120 min), seguido de dimetilsufóxido para romper a célula, e a extração com uma solução de acetona/metanol (7:3, v/v).

O processo de extração de carotenoides com solventes orgânicos é um dos métodos mais utilizados. Park, Kim e Chu (2007) relataram que a extração do carotenoide presente na parede celular do microrganismo depende da habilidade de permeabilização do solvente através da parede celular e da solubilidade dos carotenoides com o solvente utilizado. Estes autores testaram cinco solventes para a ruptura das células de *R. glutinis*, encontrando β-caroteno, toruleno e torularrodina no extrato. A mistura dos solventes dimetilsufóxido (DMSO), éter de petróleo e acetona mostrou-se eficiente, gerando máxima extração quando comparada aos solventes individuais. Como os carotenoides se encontram fortemente associados às células, Valduga et al. (2009a) testaram 11 métodos diferentes de rompimento celular e extração com solventes, com a intenção de maximizar a extração dos pigmentos. Foi constatado que quando se utilizou a combinação nitrogênio líquido e DMSO para ruptura celular e extração com mistura acetina e metanol (7:3), obteve-se a maior recuperação de carotenoides da levedura *S. salmonicolor*.

2.3 Café

O café é uma das bebidas mais populares do mundo e tem crescido constantemente, em importância comercial, durante os últimos anos (DIAS et al., 2014). A palavra café tem origem na palavra árabe *quahweh*. Hoje, sua popularidade é identificada por vários termos em diferentes países, como *cafe* (françês), *caffe* (italiano), *kaffee* (alemão), *koffie* (holandês), café (português) e *coffee* (inglês) (MURTHY et al., 2012). A província de Kaffa, na Etiópia, é considerada o hábitat original do *Coffea arabica* (arábica). A outra espécie comercialmente utilizada é o *Coffea canephora* (robusta), nativa da África Central.

O café, pertencente à família botânica *Rubiaceae*, tem cerca de 500 gêneros e mais de 6.000 espécies, mas o gênero *Coffea* é o membro mais importante, em termos econômicos (ORGANIZAÇÃO INTERNACIONAL DO CAFÉ - OIC, 2013). O cultivo do café está localizado, principalmente, nos trópicos, onde não fica exposto ao frio intenso, e em altas altitudes, evitando temperaturas muito elevadas (SCHWAN; WHEALS, 2003).

Os principais países produtores de café são Brasil, Vietnã, Colômbia, Indonésia, Etiópia e Índia, com 72,5% da produção total mundial na safra de 2010 (ICO, 2011). Com a ampla disseminação de cultivo do café em todo o mundo, hoje o Brasil é o segundo maior produtor e exportador mundial de café (ICO, 2012).

2.3.1 Composição do café

O fruto do café é constituído, normalmente, por duas sementes envolvidas por uma fina membrana conhecida como película prateada, que são protegidas por uma camada rígida chamada pergaminho, no qual está aderido

firmemente o mesocarpo mucilaginoso (mucilagem), seguido pela polpa, que é coberta pela casca (exorcarpo), que protege o fruto (Figura 4) (ESQUIVEL; JIMÉNEZ, 2012).

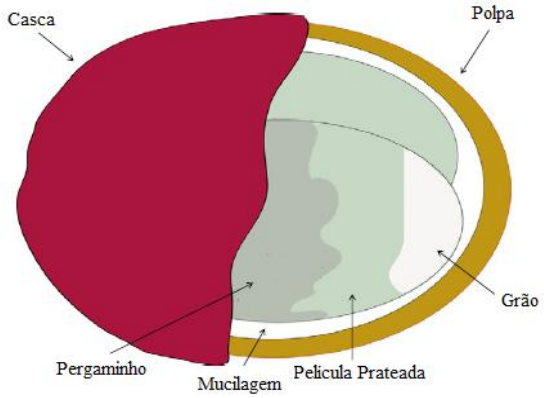

Figura 4 Camadas no fruto do café
Fonte: Esquivel e Jiménez (2012)

A camada de mucilagem está localizada entre a polpa e o pergaminho e representa 5% do peso seco do fruto (SCHWAN; WHEALS, 2003), apresentando, em sua composição, água, açúcares, substâncias pécticas, ácidos orgânicos (ELÍAS, 1978), holocelulose, lipídeos e proteínas. Dentre esses componentes, as substâncias pécticas (ARUNGA, 1982) estão entre as mais importantes, correspondendo a 30% dos polissacarídeos constituintes da mucilagem (AVALLONE et al., 2000).

2.3.2 Processamento do café

Existem, no Brasil, três tipos de processamento: via seca, que produz o café natural, e vias semisseca e úmida, que produzem o café descascado, despolpado e desmucilado. No processamento via seca, o café é seco com todas as suas partes constituintes. Já pelos processamentos vias semisseca e úmida, de

acordo com a parte removida do fruto de café, originam-se: i) cereja descascado, a casca e parte da mucilagem dos frutos são retirados mecanicamente e as sementes são secas com o restante da mucilagem e o pergaminho; ii) café despolpado, em que a casca e a mucilagem dos frutos são retiradas mecanicamente e as sementes são submetidas ao processo de fermentação para a retirada do restante da mucilagem que ficou aderida ao pergaminho e iii) café desmucilado, que é obtido com a retirada total da casca e da mucilagem por meio de máquinas conhecidas como desmuciladores (DIAS et al., 2014).

O método de processamento via semisseca é uma variação do processamento via úmida. Nesse tipo de processamento, os frutos são descasacados, despolpados ou desmucilados e o processo de fermentação ocorre diretamente no terreiro (PANDEY et al., 2000).

2.4 Subprodutos do café

O café é a segunda maior *commodity* negociada no mundo e gera grande quantidade de resíduos/subprodutos, durante o processamento (MUSSATTO et al., 2011; NABAIS et al., 2008). De acordo com dados da ICO (2013), o Brasil produziu, em 2012, cerca de 50,826 milhões de sacas de café. Esta produção gerou, aproximadamente, 3 milhões de toneladas de resíduos.

Dependendo do método de processamento do café, ou seja, processo úmido, semisseco ou seco, resíduos sólidos da fermentação e torrefação, como polpa e casca, são obtidos. As cascas de café e a polpa compõem cerca de 45% do fruto e são o principais subprodutos da indústria cafeeira. Eles são utilizados para vários fins, incluindo a extração de cafeína e polifenóis (ESQUIVEL; JIMÉNEZ, 2012), devido ao alto teor de nutrientes (Tabela 2).

Tabela 2 Composição química dos subprodutos do café

Parâmetros (%)	Polpa de café	Casca de café
Celulose	63,0±2,5	43,0±8,0
Hemicelulose	11,5±2,0	7,0±3,0
Proteína	11,5±2,0	8,0±5,0
Lipídeos	2,0±2,6	0,5±5,0
Fibras totais	60,5±2,9	24±5,9
Polifenóis totais	1,5±1,5	0,8±5,0
Açúcares totais	14,4±0,9	58,0±20,0
Substâncias pécticas	6,5±1,0	1,6±1,2
Lignina	17,5±2,2	9,0±1,6
Taninos	3,0±5,0	5,0±2,0
Ácido clorogênico	2,4±1,0	2,5±0,6
Cafeína	1,5±1,0	1,0±0,5

Fonte: Murthy et al. (2012) modificado

Uma vez que mais de 50% do fruto do café não são utilizados na comercialização e, portanto, são eliminados durante o processamento, é interessante encontrar aplicações para esses resíduos. Até agora, o maior progresso foi alcançado na sua utilização para fins industriais, como a produção de energia (KONDAMUDI; MOHAPATRA; MISRA, 2008), a adsorção dos compostos (FRANCA; OLIVEIRA; FERREIRA, 2009) e a fabricação de produtos industriais, como etanol, ácido giberélico e α-amilase (BEKALO; REINHARDT, 2010; MACHADO et al., 2002; MURTHY et al., 2009).

Nos últimos anos houve um aumento na tentativa de tornar mais eficiente a utilização desses resíduos, cuja disposição no meio ambiente causa sérios problemas de poluição. Com a inovação biotecnológica na área de enzimas e tecnologia das fermentações, novas perspectivas estão sendo criadas (DIAS et al., 2014), como a possível aplicação desses resíduos como fonte de carbono em bioprocessos para a obtenção de produtos com valor agregado. Pode-se citar, como comercialmente importante, a produção de enzimas, alcoóis,

proteínas, ácidos orgânicos, aminoácidos, metabólitos secundários biologicamente ativos e compostos de aroma (MURTHY et al., 2012) que são utilizados nas indústrias farmacêutica, de cosméticos e de alimentos (LAUFENBERG; KUNZ; NYSTROEM, 2003; WYMAN, 2003).

Uma das primeiras abordagens sobre a aplicação da polpa e casca de café foi com a produção de enzimas pectinase, tanase e cafeinase. A produção de tanase, utilizando casca de café com *Paecilomyces variotii* em condições otimizadas, foi estudada por Battestin e Macedo (2007). A produção da enzima foi 8,6 vezes maior, comparada com a produção em substrato sintético. A utilização de subprodutos do café para a produção de enzimas, como amilase, protease e xilanase, foi realizada utilizando-se os fungos *N.crassa*, *A. oryzae*, *Penicillium* sp. e *A. niger* (MURTHY; NAIDU, 2010).

A utilização de resíduos como casca de café, folhas e borra foi avaliada no cultivo de cogumelos das espécies *Lentínula edodes*, *Pleurotus* spp. e *Flammulina velutipes* (MURTHY; MANONMANI, 2008). Machado et al. (2002) relataram a produção de giberelinas (hormônio de plantas) em fermentação submersa (FSm) e em estado sólido (FES), utilizando casca de café como fonte de carbono em cinco cepas de *Gibberella fujikuroi*. A produção de ácido giberélico obtida foi de 1100 mg/kg.

Os produtos agroindustriais são boas fontes de compostos fenólicos e têm sido utilizados como fontes de antioxidantes naturais (FKI; ALLOUCHE NANDSAYADI, 2005). A polpa de café tem sido explorada na obtenção de polifenóis (SERA et al., 2000). Utilizando subproduto do café (película de prata), Machado (2009) avaliou a capacidade de sete diferentes isolados de fungos *Aspergillus*, *Mucor*, *Penicillium* e *Neurospora* em crescer e liberar compostos fenólicos sob condições de cultivo em estado sólido, objetivando a detoxificação biológica deste resíduo. De acordo com os autores, *Penicillium purpurogenum*, *A. niger* AA20, *Neurospora crassa* e *Mucor* liberaram altas

quantidades de compostos fenólicos (entre 2,28 e 1,92 g/L) da película de prata e, dessa forma, teve sucesso na detoxificação do resíduo de café, o que seria vantajoso para a sua eliminação no meio ambiente.

Medeiros et al. (2003) reportaram que há grande potencial no uso de polpa e casca de café como substratos para produção de compostos aromáticos produzidos por fermentação em estado sólido, utilizando duas diferentes cepas de *Ceratocystis fimbriata.*

2.4.1 Polpa de café

A polpa do café é o primeiro subproduto obtido durante o processamento e representa 29% do peso seco de toda a baga (Figura 5). A polpa é obtida durante o processamento úmido do café e a cada duas toneladas de café produzidas é obtida uma tonelada de polpa (ROUSSOS et al., 1995). A polpa de café é essencialmente rica em carboidratos, proteínas e minerais (principalmente potássio) e também contém apreciáveis quantidades de taninos, polifenóis e cafeína (BRESSANI; ESTRADA; JARQUIN, 1972). Os componentes orgânicos presentes na polpa (em peso seco) incluem taninos 1,80%-8,56%, substâncias pécticas totais 6,5%, açúcares redutores 12,4%, açúcares não redutores 2,0%, cafeína 1,3%, ácido clorogênico 2,6% e ácido cafeínico total 1,6% (MURTHY et al., 2012).

2.4.2 Casca de café

Cascas de café são obtidas quando as bagas de café são processadas pelo método seco. A casca envolve os grãos e representa, aproximadamente, 12% do peso seco da baga (MURTHY et al., 2012). Cerca de 0,18 toneladas de casca são produzidas a partir de uma tonelada de frutos de café (ADAMS; DOUGAN,

1981). Casca de café contém 15,0% de umidade, 5,4% de cinzas, 7,0% de proteína, 0,3% de lipídeos e 72,3% de carboidratos (GOUVEA et al., 2009), além de 24,5% de celulose, 29,7% hemiceluloses e 23,7% da lignina (BEKALO; REINHARDT, 2010).

Figura 5 A) Polpa e B) casca de café
Fonte: Murthy et al. (2012) modificado

3 CONSIDERAÇÕES FINAIS

A economia brasileira é uma das mais importantes do mundo baseadas na agricultura, produzindo e exportando café, cana-de-açúcar, soja, mandioca e frutas, entre outros. Entretanto, o processamento desses produtos agrícolas gera uma grande quantidade de resíduos.

Os resíduos agroindustriais podem apresentar uma fonte de nutrientes, de carboidratos simples e complexos que poderia ser utilizada em processos de fermentação. A indústria cafeeira no Brasil gera, aproximadamente, 3 milhões de toneladas de subprodutos que são ricos em carboidratos, proteínas, pectinas e compostos bioativos, como polifenóis, além de serem considerados recursos renováveis e baratos.

Diante do exposto, neste trabalho aborda-se a produção de moléculas bioativas (pigmentos) por microrganismos, que irão utilizar como substratos resíduos do processamento do café (polpa e casca), o que representa um novo desafio no manejo desses subprodutos. Torna-se necessária a seleção de microrganismos capazes de utilizar estes resíduos como substratos para a produção de biomassa e de carotenoides. Além disso, buscam-se a otimização do meio de produção para melhor aproveitamento dos componentes do meio de fermentação e avaliar as condições de cultivo e os principais parâmetros que interferem na produção, visando boa produtividade e fácil recuperação do produto.

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SEGUNDA PARTE - ARTIGOS

ARTIGO 1

Use of coffee processing by-products for the production of carotenoids by yeasts and bacteria

Versão preliminar a ser submetida ao periódico *Jounal of Applied Microbiology*, preparada de acordo com suas normas de publicação.

Use of coffee processing by-products for the production of carotenoids by yeasts and bacteria

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Abstract

Aim: Use of the byproducts coffee from dry and semi-dry process, as carbon source for carotenoid production by yeasts and bacteria. Test different combinations of organic solvents for recovery of intracellular carotenoid and optimize production.

Methods and results: The production of carotenoids by *Rhodotorula mucilaginosa* CCMA 0156, *R. mucilaginosa* CCMA 0340 and *Dermacoccus nishinomiyaensis* CCMA 0685 was evaluated in coffee pulp extract (PE) and coffee husk extract (HE). Acetone: methanol (7: 3 v/v) acetone: petroleum ether (1: 1 v/v) ethyl acetate: petroleum ether (1: 1 v/v), were solvents used for the extraction and recovery of intracellular carotenoids. A Plackett–Burman design was used, followed by a Central Composite Design, to optimize the production of specific carotenoids by *R. mucilaginosa* CCMA 0156 in coffee pulp extract and husk extract medium. Maximum concentration of specific carotenoids (361.29±36.0 µg g^{-1}) was obtained in 6.68 % pulp extract, 10.04 g l^{-1} peptone and 3 g l^{-1} yeast extract. Maximum production specific carotenoids in 8.36 % husk extract medium containing 6.36 g l^{-1} glucose and 3.68 g l^{-1} peptone, was 296.58±13.2 µg g^{-1}.

Conclusion: The pulp and coffee husks are potential substrates for the production of carotenoids by micro-organisms. The three extraction methods were efficient in the recovery of intracellular carotenoids. After the optimization process, carotenoid production in PE and HE increased 4.43-fold and 3.08-fold, respectively. These results showed that the statistical experimental design offers a practicable approach to the implementation of optimization of carotenoid production.

Significance and Impact of the Study: Countries whose economy is based on agricultural activities generate a great deal of liquid and solid waste. Thus, it is

important to develop new alternatives for using this waste rather than disposing it in the environment. The production of carotenoid is one such alternative. This is the first report on the use of coffee processing waste as culture media for the production of carotenoid by micro-organisms.

Keywords: Pigments. *Rhodotorula mucilaginosa*. *Dermacoccus nishinomiyaensis*. Coffee pulp. Coffee husk. Surface response methodology.

Introduction

The carotenoids are a group composed of liposoluble natural pigments widely distributed in environment, with a great diversity of structures and functions. They are responsible for the yellow, orange and red found in many plants, animals and micro-organisms. (Botella-Pavía & Rodríguez-Concepción, 2006; Maldonade et al., 2007; Valduga et al., 2011).

Nowadays, the carotenoids are desirable compounds in several industries such as chemical, pharmaceutical, food and cosmetics. These pigments have attracted the attention of industry and researchers, because they can act as vitamin A precursors and also feature antioxidant properties. Carotenoids are natural antioxidants that can relieve chronic diseases, retard aging and reduce various disease stages. (Park et al., 2007; Das et al., 2007; Maldonade et al., 2008).

Most of carotenoids are produced by chemical synthesis or extraction from the plants (Dufossé et al., 2005). These ways are limited by low productivity which results in high production costs. That situation gives rise to production regulated by chemical synthesis methods for obtaining carotenoids as additives (Marova et al., 2010).

In this context, the biosynthesis of carotenoids by microbial fermentation has emerged as an alternative (Park et al., 2007; Valduga et al.,

2009, 2011). Microbial production by fermentation can be performed using inexpensive substrates such as agricultural residues. These residues are sources of carbon, nitrogen and other elements necessary for the growth and production of the microorganism (Nigam et al. 2009; Lopes et al., 2013).

The industrial production of natural carotenoids by microbial fermentation is expanding (Monks et al., 2012; Mata-Gómez et al., 2014). Yeasts and bacteria produce carotenoids which are synthesized and stored inside the cells, so extraction processes are needed to recover the product of interest (Valduga et al., 2009). Downstream treatment is a major challenge to recover intracellular pigments. Many studies have been done on finding more efficient recoveries, reduced costs, high performance and productivity of microbial carotenoids (Valduga et al., 2009; Michelon et al., 2012; Mata-Gómez et al., 2014).

The carotenoids are liposoluble, so extraction can be carried out with organic solvents such as acetone, petroleum ether, hexane, chloroform, ethanol, and methanol (Valduga et al., 2009). Some studies have reported the use of these (isolated or combined) solvents, comparing their extraction capacity of carotenoids from microbial cells. Some solvents, when used together to break through the cells, can show synergistic interactions which results in higher yields of carotenoids (Mendes Pinto et al., 2001; Lim et al., 2002; An e Choi, 2003; Park et al., 2007; Valduga et al., 2009; Monks et al., 2012).

In this context, this study aimed to use by-products of coffee processing (pulp and coffee husk) as only carbon source for production of carotenoids by yeasts and bacteria. Furthermore, evaluate different combinations of organic solvents for extraction and recovery of intracellular carotenoids and optimize production.

Materials and methods

By-products and Pretreatment

Coffee husk and pulp from dry and semi-dry process respectively of the coffee beans (*Coffea arabica* L., variety red Catuaí 99) was supplied by a coffee-producing unit located in the southern area of the state of Minas Gerais (Brazil). When obtained, the materials were immediately frozen at -20°C. The coffee husk and pulp was dried at 65°C until it reached a constant weight (AOAC) (1995). Later, were ground in a Willey mill (1.0 mm) and stored in plastic flasks. The substrates were pretreated individually with 0.06 % (w/v) KOH. Following this, 50 g of each substrate dry and milled was resuspended in 1 l of distilled water containing KOH and sterilization was carried out at 121 º C for 30 min (Machado et al., 2002). The extracts were filtered with Whatman Nº. 1 and used for fermentation studies.

Physicochemical analysis

Coffee husk and pulp (*in nature* and pretreated) were characterized as to crude protein content, total soluble carbohydrates and pH. The determination of crude protein and the content of dry matter (DM) definitive were according to the methods recommended by the Association of Official Agricultural Chemists (AOAC, 1995). The total soluble carbohydrates (CHOs) were determined by phenol-sulfuric method described by Dubois et al. (1956) with some modifications. The pH was determined by using a digital potentiometer (Digimed analytical, Model DM22).

Screening micro-organisms and carotenoids production

The strains used in this study are belonging to the Culture Collection of Agricultural Microbiology (CCMA) of Department of Biology, Federal University of Lavras, Brazil. Two orange color yeasts, *Rhodotorula mucilaginosa* CCMA 0156 and *R. mucilaginosa* CCMA 0340 and one bacteria of yellow color, *Dermacoccus nishinomiyaensis* CCMA 0685 were tested for production of carotenoids in coffee pulp extract and husk extract. The strain that had the highest production of carotenoids was submitted to an Experimental Design.

The production of carotenoids was in submerged fermentation using husk extract and coffee pulp extract (50 g l^{-1} each one). For production comparison, it was also tested in synthetic medium YM (Yeast Malt Extract) (g l^{-1}) (w/v): 3.0 yeast extract, 3.0 malt extract, 5.0 peptone and 10.0 glucose. The micro-organisms were prepared in 10 ml tubes of YM medium for 24 h, after that, they were transferred to 250 ml Erlenmeyer flasks with 90 ml of the same medium and incubated for 48 h. Before inoculation in the fermentation media, the cells were centrifuged at 9000 rpm, 4 ° C for 10 min and washed 2x with distilled water.The flasks were inoculated with 10^7 ufc ml^{-1}. The cultivations were carried out in a shaker under 28 °C temperature and agitation in 500 ml Erlenmeyer flasks containing 300 ml of medium and incubated at 28 °C, 160 rpm for 4 days in the dark. The experiments were conducted in triplicate.

Experimental Design

Experimental design technique was used to study the effects of medium composition. The effects of the composition of the culture medium and the fermentation conditions were assessed by a Plackett–Burman Design (*Screening*

Design) with twelve assays and three central points (Rodrigues and Iemma, 2009). Experimental variables and their corresponding levels are summarized in Table 1.

Table 1 Variables and levels used in the Plackett-Burman planning type for the screening means design with coffee pulp extract and husk extract for the production of carotenoids by *Rhodotorula mucilaginosa* CCMA 0156

Variables	Codes	Levels		
		-1	0	+1
pH	X_1	4	6	8
Glucose (g l^{-1})	X_2	0	2	4
Yeast extract (g l^{-1})	X_3	0	1	3
Malt extract (g l^{-1})	X_4	0	1	3
Ammonium sulfate (g l^{-1})	X_5	0	1	2
Tween 80 (%)	X_6	0	0,5	1
Peptone (g l^{-1})	X_7	0	2	5
Pulp extract or husk extract (%)	X_8	1	3	5

Based on the results obtained in the Plackett–Burman design, a central composite design (CCDR) based on the RSM was employed to illustrate the response behavior in the optimum region, including a 2^3 design, three replications of the center points, and six axial points. The CCDR matrix with independent variables studied in the fermentation with coffee pulp extract and husk extract are shown in the Tables 2 and 3. The response or dependent variables studied were total carotenoids (µg l^{-1}), specific production of carotenoids (µg g^{-1}) and biomass (g l^{-1}).

Table 2 CCDR matrix (real and coded values) of three variables (pulp extract, peptone and yeast extract) used in the production of carotenoids by *Rhodotorula mucilaginosa* CCMA 0156 in coffee pulp extract, 28 ° C, 160 rpm in the dark for 5 days

Assays	Pulp extract X_1	Peptone X_2	Yeast extract X_3
1	-1 (3%)	-1 (2 g l^{-1})	-1 (1 g l^{-1})
2	1 (7%)	-1 (2 g l^{-1})	-1 (1 g l^{-1})
3	-1 (3%)	1 (8 g l^{-1})	-1 (1 g l^{-1})
4	1 (7%)	1 (8 g l^{-1})	-1 (1 g l^{-1})
5	-1 (3%)	-1 (2 g l^{-1})	1 (5 g l^{-1})
6	1 (7%)	-1 (2 g l^{-1})	1 (5 g l^{-1})
7	-1 (3%)	1 (8 g l^{-1})	1 (5 g l^{-1})
8	1 (7%)	1 (8 g l^{-1})	1 (5 g l^{-1})
9	-1,68 (1.64%)	0 (5 g l^{-1})	0 (3 g l^{-1})
10	1,68 (8.36%)	0 (5 g l^{-1})	0 (3 g l^{-1})
11	0 (5%)	-1,68 (0.04 g l^{-1})	0 (3 g l^{-1})
12	0 (5%)	1,68 (10.04 g l^{-1})	0 (3 g l^{-1})
13	0 (5%)	0 (5 g l^{-1})	-1,68 (0.36 g l^{-1})
14	0 (5%)	0 (5 g l^{-1})	1,68 (6.36 g l^{-1})
15	0 (5%)	0 (5 g l^{-1})	0 (3 g l^{-1})
16	0 (5%)	0 (5 g l^{-1})	0 (3 g l^{-1})
17	0 (5%)	0 (5 g l^{-1})	0 (3 g l^{-1})

Table 3 CCDR matrix (coded and real values) of three variables (glucose, peptone and husk extract) used in the production of carotenoids by *Rhodotorula mucilaginosa* CCMA 0156 in coffee husk extract, 28 ° C, 160 rpm in the dark for 5 days

Assays	Glucose X_1	Peptone X_2	Husk extract X_3
1	-1 (1 g l^{-1})	-1 (1 g l^{-1})	-1 (3%)
2	1 (5 g l^{-1})	-1 (1 g l^{-1})	-1 (3%)
3	-1 (1 g l^{-1})	1 (3 g l^{-1})	-1 (3%)
4	1 (5 g l^{-1})	1 (3 g l^{-1})	-1 (3%)
5	-1 (1 g l^{-1})	-1 (1 g l^{-1})	1 (7%)
6	1 (5 g l^{-1})	-1 (1 g l^{-1})	1 (7%)
7	-1 (1 g l^{-1})	1 (3 g l^{-1})	1 (7%)
8	1 (5 g l^{-1})	1 (3 g l^{-1})	1 (7%)
9	-1,68 (0.36 g l^{-1})	0 (2 g l^{-1})	0 (5%)
10	1,68 (6.36%)	0 (2 g l^{-1})	0 (5%)
11	0 (3 g l^{-1})	-1,68 (0.32 g l^{-1})	0 (5%)
12	0 (3 g l^{-1})	1,68 (3.68 g l^{-1})	0 (5%)
13	0 (3 g l^{-1})	0 (2 g l^{-1})	-1,68 (1.64%)
14	0 (3 g l^{-1})	0 (2 g l^{-1})	1,68 (8.36%)
15	0 (3 g l^{-1})	0 (2 g l^{-1})	0 (5%)
16	0 (3 g l^{-1})	0 (2 g l^{-1})	0 (5%)
17	0 (3 g l^{-1})	0 (2 g l^{-1})	0 (5%)

The preparation of the inoculum *Rhodotorula mucilaginosa* CCMA 0156 was as described in the previous section. The starter culture was 10.0% (w/v) inoculated by batch fermentation. The cultivations were carried in a shaker under temperature control and agitation in 250 ml Erlenmeyer flasks containing 150ml of medium, inoculated with 10^7 cél ml^{-1} and incubated at 28 ° C, 160 rpm for 5 days in the dark.

Validation of the experimental design

The validations experiments carotenoids production was performed both for coffee pulp extract (PE) and coffee husk extract (HE). The validation was carried out under the optimized conditions indicated by the STATISTICA 8.0 software. The model was tested in triplicate assays using 150 ml of culture medium, stirring at 160 rpm, 28 ºC, pH 5, in the dark for 5 days. The composition of the medium in pulp extract: X_1 - pulp extract (6.68 %), X_2 - peptone (10.04 g l^{-1}) and X_3 - yeast extract (3 g l^{-1}); glucose (2 g l^{-1}) and tween 80 (0.5 %) fixed factors . In husk extract: X_1 - glucose (6.36 g l^{-1}), X_2 - peptone (3.68 g l^{-1}) and X_3 - husk extract (8.36 %) and tween 80 (0.5 %) fixed factor.

Extraction and recovery of carotenoids

The carotenoids produced by the three microorganisms were recovered by three different extraction methods, according to the methodology proposed by Valduga et al. (2009a), adapted. The solvent mixtures used were as follows: method 1 - acetone: methanol (7: 3, v/v); method 2 - acetone: petroleum ether (1: 1, v/v) and method 3 - ethyl acetate: petroleum ether (1: 1, v/v). The cell disruption step was similarly for all three methods. Previously biomass recovered from the fermentation medium was centrifuged (9,000 × g, 4 ° C, 10 min) and dried in oven at 65 ° C for 24 h. Then macerated in liquid N_2 and added to 2 ml of dimethylsulfoxide (DMSO) and heated in a water bath at 55 ° C / 30 min. After this time, 2 ml of mixed solvent were added and centrifuged (5,000 × g, 4 ° C, 10 min). The supernatant was separated, and successive extractions were carried out until both solvent and cells remain colorless. The solvent was evaporated in a nitrogen gas atmosphere and the pigments were solubilized in 5 ml methanol (method 1) and 5 ml of petroleum ether (method 2

and 3). Experiment design was used method 1 as described above for recovery and extraction of carotenoids.

Determination of total carotenoids

The absorbance of the sample after the extraction was measured in a spectrophotometer (Biospectro, Model SP-220). The concentration of total carotenoids was estimated by the maximum absorbance at 450 nm, using the equation described by Davies (1976). The coefficient of absorbance used was that referent to β-carotene: $E^{1\%}_{1\ cm} = 2592$, for petroleum ether and $E^{1\%}_{1\ cm} = 2550$, for methanol (Silva et al., 2004). Concentration of carotenoids was expressed in terms of total carotenoids (μg l^{-1}) and specific production of carotenoids (μg g^{-1}). The specific production of carotenoids represents the total concentration of carotenoids (μg) in relation to the biomass of dried yeast or bacteria obtained in 1 L of a fermented medium (Davies 1976).

Determination of biomass, reducing sugar, pH and count

After extraction of carotenoids, the cells were washed with distilled water and centrifuged at 5,000×g for 10 min at 4°C. The biomass was quantified through drying at 65°C until a constant weight. The procedure used for quantification of reducing sugar was the DNS (3,5-dinitrosalicylic) methodology as described by Miller (1959) and the staining intensity was measured by spectrophotometer (Biospectro, Model SP-220) at a wavelength of 540 nm. The pH of the culture media was determined using a digital pH meter (Digimed analytical, Model DM22). Cell growth was evaluated by counting in Neubauer Chamber (yeast) and counting on plate (bacteria).

Statistical analysis

The results obtained from all performed experiments were subjected to analysis of variance (ANOVA), applying entirely randomized design, with factorial arrangement 3x3 (3 extraction methods and 3 culture media), using Sisvar® 4.5 (Lavras, Brazil) software. The F-test was used to determine significant effects of the treatments, and Scott-Knott test was used to compare the average of replicates at a 5 % level of significance. The results obtained from experimental design were analyzed using the statistical software Statistica 8.0 (Stat soft. Inc.®, Tulsa, ok). The statistical significance of the model was determined using Fisher's test. The fit of the regression model was checked by the adjusted coefficient of determination R^2. Response surface plots were generated by the same software. Finally, the location of the optimum condition was calculated by differentiation of the quadratic model.

Results

Characterization of by-products

In our study were quantified the contents of carbohydrates, protein, dry matter and pH in the samples of pulp and coffee husks *in nature* and pretreated (extracts) (Table 4). The pulp and coffee husks are naturally acidic, the pH in this study ranged from 4.5 to 4.7, in the extracts *in nature* pulp and coffee husk, respectively. After the pretreatment with KOH, the pH of the extracts were slightly changed to 5.0 (pulp extract) and 5.7 (husk extract). Table 4 show the chemical composition of these by-products is similar both *in nature* and pretreated. However, comparing the dry matter content in the samples in natura and pretreated there was a great loss after pretreatment, as expected.

Table 4 Physicochemical composition of pulp and coffee husk *in nature* and after pretreatment

Parameters	*in nature*		pretreated	
	Coffee pulp	Coffee husk	Coffee pulp	Coffee husk
pH	4.5	4.7	5.0	5.7
Dry matter (g Kg^{-1})	1181.64	982.39	242.11	305.07
Concentration (g Kg^{-1} MS)				
Crude protein	173.7	137.23	171.07	133.79
Total soluble carbohydrates	156.8	136.15	120.09	100.7

Extraction and quantification of carotenoids

The production of carotenoids *Rhodotorula mucilaginosa* CCMA 0156, *R. mucilaginosa* CCMA 0340 and *Dermacoccus nishinomiyaensis* CCMA 0685 was evaluated in three different fermentation media (YM, PE, and HE) and recovery of intracellular carotenoid was performed with three different combinations of solvents. The solvents were tested, and their extraction yields, expressed as µg l^{-1} of total carotenoids extracted and micrograms per gram of specific carotenoids, are listed in Table 5. All tested combinations of solvents showed to be efficient in the extraction of carotenoids for the three microorganisms, with diferrence in production rates. The three media and the three methods were statistically significant (p<0.05) for each variable analyzed (total carotenoids, specific carotenoids and biomass) and strain.

Table 5 Concentration of total carotenoids, specific and biomass produced by strains *Rhodotorula mucilaginosa* CCMA 0156, *Rhodotorula mucilaginosa* CCMA 0340 and *Dermacoccus nishinomiyaensis* CCMA 0685 in the different culture medium YW, PE and HE and extraction methods 1, 2 and 3

TC (µg l^{-1})									
Microorganisms	*R. mucilaginosa* CCMA 0156			*R. mucilaginosa* CCMA 0340			*Dermacoccus nishinomiyaensis* CCMA 0685		
Method*\Medium**	YM	PE	HE	YM	PE	HE	YM	PE	HE
1	251.3aA	331.2aA	255.0aA	306.5aA	160.5bA	137.6bA	16.1aA	41.6bA	9.3aA
2	184.7aA	269.0aA	215.0aA	421.9aA	106.2bB	218.2abB	16.2aA	19.0aB	11.2aA
3	195.2aA	259.2aA	250.4aA	128.8aB	186.8aA	105.9aA	30.0aB	50.8aA	3.4bB
SC (µg g^{-1})									
Microorganisms	*R. mucilaginosa* CCMA 0156			*R. mucilaginosa* CCMA 0340			*Dermacoccus nishinomiyaensis* CCMA 0685		
Method*\Medium**	YM	PE	HE	YM	PE	HE	YM	PE	HE
1	57.0bA	81.4aA	96.2aA	90.8aA	62.4aA	74.3aA	7.0aA	17.6bAB	8.0aA
2	74.3aA	69.2aA	79.4aA	130.3aA	57.2bA	119.6aB	6.5aA	13.2aA	7.4aA
3	62.7aA	70.4aA	86.1aA	42.61aB	80.9aA	81.7aAB	13.1aA	26.9bB	3.5aB
B (g l^{-1})									
Microorganisms	*R. mucilaginosa* CCMA 0156			*R. mucilaginosa* CCMA 0340			*Dermacoccus nishinomiyaensis* CCMA 0685		
Method*\Medium**	YM	PE	HE	YM	PE	HE	YM	PE	HE
1	4.4aA	4.0aA	2.6bA	3.3aA	2.5aA	1.8aA	2.3aA	2.3aA	1.1bA
2	2.4aB	3.8bA	2.7abA	3.2aA	1.8bA	1.8bA	2.4aA	1.4bA	1.5bA
3	3.1aB	3.6aA	3.0aA	3.0aA	2.3aA	1.3bA	2.3aA	1.8aA	1.0bA

*Means followed by the same capital letters, followed in columns and ** mean the same lowercase letters, in the lines, do not differ by the scott knott test ($p < 0.05$) for each strain and analyzed variable (tc - total carotenoids, sc - specify carotenoids and b - biomass). methods: 1 - acetone: methanol (7: 3 v/v); 2 - acetone: petroleum ether (1: 1 v/v) and 3 - ethyl acetate: petroleum ether (1: 1 v/v). ym - sinthetic medium; pe - coffee pulp extract; he - coffee husk extract

The extraction yields ranges from 42.61 µg g^{-1} to 130.36 µg g^{-1} from the synthetic medium (YM) by *R. mucilaginosa* CCMA 0340, recovered by three methods (ethyl acetate / petroleum ether (1: 1, v/v)) and 2 (acetone / petroleum ether (1: 1, v/v)), respectively, representing a difference of 67%. The highest biomass production observed between microorganisms and the three media was the strain *R. mucilaginosa* CCMA 0156. However, the maximum production of specific carotenoids (96.26 µg g^{-1}) from this yeast in husk extract was not accompanied by maximum production biomass 2.64 g l^{-1}, as observed for *R. mucilaginosa* CCMA 0340 and the bacteria. *Dermacoccus nishinomiyaensis* CCMA 0685 produced less carotenoids than the yeasts. Comparing the three medium, the bacteria has the highest production of carotenoids 26.9 µg g^{-1} in in pulp extract and method 3 recovered. The largest difference observed (56%) in the production of carotenoids by the cultivation of the bacterium was husk extract 3.5 µg g^{-1} recovered by the method 3 e 8 µg g^{-1} (método 1) (Tabela 5).

Fermentation parameters

As shown in Fig.1, comparing the three micro-organism and three medium of culture in all the time due fermentations, a carotenoids production was accompanied by the increase in biomass production and decrease of reducing sugars. *R. mucilaginosa* CCMA 0156 presented at the end of 96 h fermentation, the maximum total production carotenoids (154.29 µg l^{-1}) and largest biomass production (5.64 g l^{-1}) growing in pulp extract (Fig. 1B). However, *R. mucilaginosa* CCMA 0340 had a production 84.83% (23.4 µg l^{-1}) less than *R. mucilaginosa* CCMA 0156, in pulp extract (Fig. 1E). *Dermacoccus nishinomiyaensis* CCMA 0685 had a lower carotenoids production (average 5.8 µg l^{-1}) in three culture media (Fig. 1G, 1H and 1I) as well as lower biomass production (1.65 g l^{-1}) in husk extract (Figure 1I).

The two strains *R. mucilaginosa* CCMA 0156 and *R. mucilaginosa* CCMA 0340 consumed faster this sugar between synthetic, in which the consumer was 84% and 87%, respectively, than in pulp extract and husk extract in which each of consumption yeast was around 70%. In the same way, the bacteria had higher consumption in synthetic medium (44%) than in pulp extract (22%) and husk extract (18%).

The pH was monitored throughout the proving time in medium diferent with three micro-organism (Figure 1). The pH evolution during production of the carotenoids in synthetic medium was similar to three strains. The first 48 h there was a decrease from pH initial and during intense cell carotenogenesis phase pH increased. That increase after the first 48 h was also observed in pulp extract and husk extract for the three micro-organism during production of carotenoids (Fig. 1).

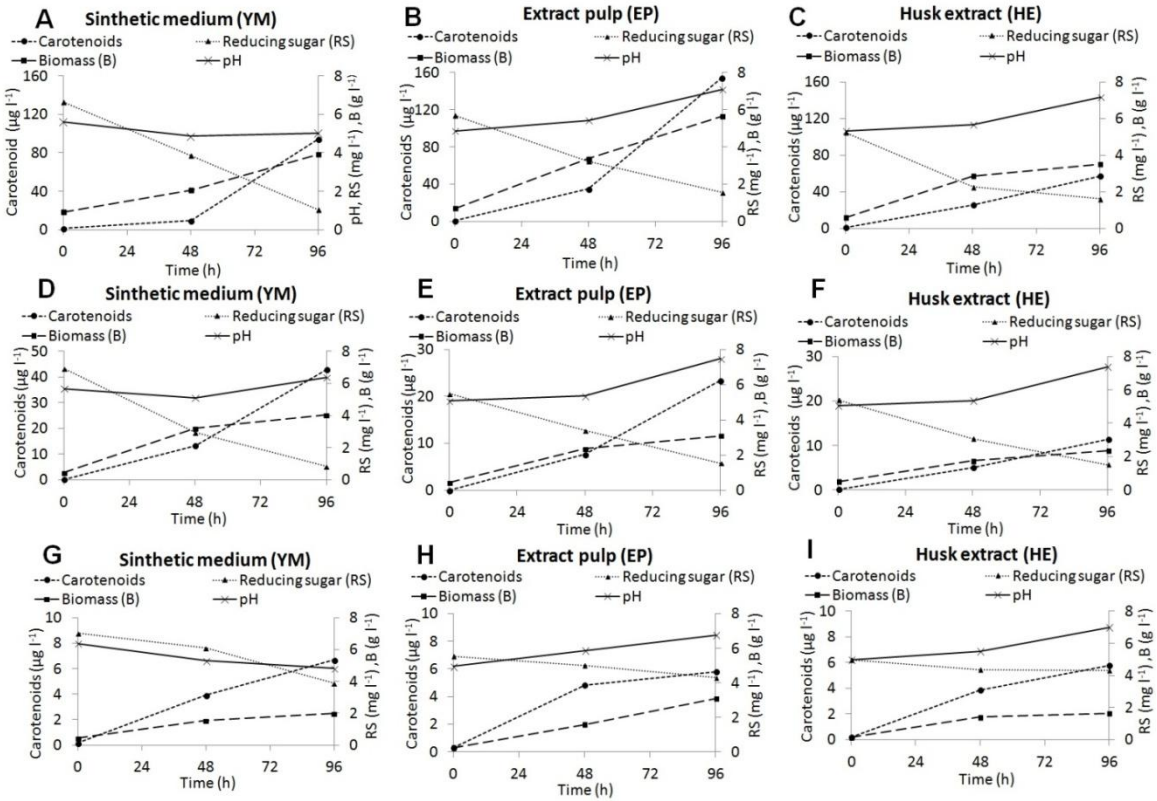

Figura 1 Kinetics of the fermentation, pH, biomass and carotenoids production and reducing sugar during 96 h in the three culture media: Sinthetic medium – YM; Extract pulp – EP and Husk extract – HE. *Rhodotorula mucilaginosa* CCMA 0156 (1A, 1B, and 1C); *R. mucilaginosa* CCMA 0340 (1D, 1E, and 1F) and *Dermacoccus nishinomiyaensis* CCMA 0685 (1G, 1H, and 1F)

Plackett–Burman Design

The Plackett–Burman (PB) experimental design determined the independent factors that had a greater influence on specific carotenoid production. These results had used to determine regression coefficients for the specific carotenoid response. Seven independent variables (X_1: pH; X_2: Glucose; X_3: Yeast extract; X_4: Malt extract; X_5: Ammonium sulfate; X_6: Tween 80; X_7: Peptone) were evaluated in experiments with coffee pulp extract (X_8) and husk extract (X_8). However, these factors influenced differently each experiment. The analysis of the PB to pulp extract showed that at 90% significance level, only yeast extract, peptone and pulp extract were significant ($p < 0.10$) in the production of specific carotenoids by *Rhodotorula mucilaginosa* CCMA 0156. In the experiment with husk extract, among the variables tested that at 99% significance level, glucose, peptone and husk extract were statistically significant ($p < 0.01$) in the production of specific carotenoids.

Optimization of Carotenoid Production

Pulp extract, peptone and yeast extract were the three independent variables evaluated in relation to specific carotenoid production, in coffee pulp extract (PE) and husk extract, glucose and peptone were the three independent variables evaluated in relation to specific carotenoid production, in husk extract (HE). The results demonstrated that there were significant relationships between the variables studied for specific carotenoids production in each medium (Table 6 and Table 7).

Table 6 Model coefficients estimated by linear and quadratic regression multiples (significance of regression coefficients) of the evaluated factors (pulp extract, peptone and yeast extract) to produce specific carotenoids by *Rhodotorula mucilaginosa* CCMA 0156 responses in the central composition design in coffee pulp extract

	Regression coefficient	Pure error	*t* value	*p* value
Mean/Interc. *	325,52	28,58	11,38	0,0000*
(X_1) Pulp extract (L) *	50,26	13,43	3,74	0,0072*
(X_1)Pulp extract(Q) *	-52,34	14,79	-3,53	0,0095*
(X_2)Peptone (L)	13,45	13,43	1,00	0,3498
(X_2)Peptone (Q)	-2,83	14,79	-0,19	0,8535
(X_3)Yeast extract(L)	-12,17	13,43	-0,90	0,3946
(X_3)Yeast extract (Q) *	-35,56	14,79	-2,4	0,0472*
X_1L by X_2L	1,50	17,54	0,08	0,9341
X_1L by X_3L	-9,13	17,54	-0,52	0,6186
X_2L by X_3L	14,73	17,54	0,84	0,4285

*Significant (p<0.05). R^2=0.82. (L) linear effect and (Q) quadratic effect

Table 7 Model coefficients estimated by linear and quadratic regression multiples (significance of regression coefficients) of the evaluated factors (husk extract, peptone and yeast extract) to produce specific carotenoids by *Rhodotorula mucilaginosa* CCMA 0156 responses in the central composition design in coffee husk extract

	Regression coefficient	Pure error	*t* value	*p* value
Mean/Interc. *	213,72	19,52	10,94	0,0000*
(X_1)Glucose (L)	19,86	9,17	2,16	0,0671
(X_1)Glucose (Q)	12,94	10,10	1,28	0,2410
(X_2)Peptone (L) *	22,11	9,17	2,41	0,0467*
(X_2)Peptone (Q)	0,36	10,10	0,03	0,9725
(X_3)Husk extract(L) *	64,33	9,17	7,01	0,0002*
(X_3)Husk extract (Q)	-14,39	10,10	-1,42	0,1974
X_1L by X_2L	3,43	11,98	0,28	0,7829
X_1L by X_3L *	-30,43	11,98	-2,54	0,0386*
X_2L by X_3L	4,33	11,98	0,36	0,7280

*Significant (p<0.05). R^2=0.91. (L) linear effect and (Q) quadratic effect

The obtained results enabled us to determine regression coefficients for the specific carotenoid response. Linear and quadratic pulp extract and quadratic yeast extract; linear peptone and linear and quadratic husk extract and the linear interaction glucose by husk extract, model parameters were significant (p<0.05) at a 95% significance level for PE and HE, respectively. The following models with codified variables were built using the significant parameters (Equation 1 and 2):

$$Y_{PE} = 325.52 + 50.26X_1 - 52.34X_1^2 - 35.56X_3^2 \quad (1)$$

$$Y_{HE} = 213.72 + 22.11X_2 + 64.33X_3 - 30.43X_1X_3 \quad (2)$$

where, Y_{PE} stands for specific carotenoids production in pulp extract: X_1 is pulp extract and X_3 represents yeast extract. And Y_{HE}, production in husk extract: X_1 is glucose, X_2 is peptone, and X_3 represents husk extract. The fits of the models were confirmed by the coefficient of determination (R^2). In Pulp extract, the coefficient of determination value (R^2=0.82) indicated that 82% of the variability in the response could be explained by the model. Husk extract, the coefficient of determination value is R^2=0.91.

Three-dimensional response surface plots and was used to visualize the interaction effects of the variables related to specific carotenoid production and to determine their optimum ranges for maximum production. Figure 2 shows the response surface for the production of specific carotenoids coffee extract pulp. Plot A reveals higher specific carotenoids production for higher peptone (10.04 g l^{-1}) concentration and average amount (center point) of yeast extract (3 g l^{-1}). Plot B illustrates that higher pigment production occurred at higher peptone (10.04 g l^{-1}) concentration associated with pulp extract (6.68 g l^{-1}) concentration

near center point. Finally, Plot C shows higher production when pulp extract (6.68 g l^{-1}) concentration near center point was associated with average amount (center point) of yeast extract (3 g l^{-1}).

Figure 3 shows the response surface for the production of specific carotenoids coffee extract husk. The maximum content of specific carotenoids was obtained in the tests corresponding to the axial point (1.68) so the highest value of each variable (6.36 g l^{-1} glucose, 3.68 g l^{-1} peptone and 8.36 g l^{-1} husk extract). Husk extract and peptone concentration influenced positively the response. Plot A, B and C show that specific carotenoids production was affected by the independent variables peptone and glucose, husk extract and peptone, husk extract and glucose, respectively.

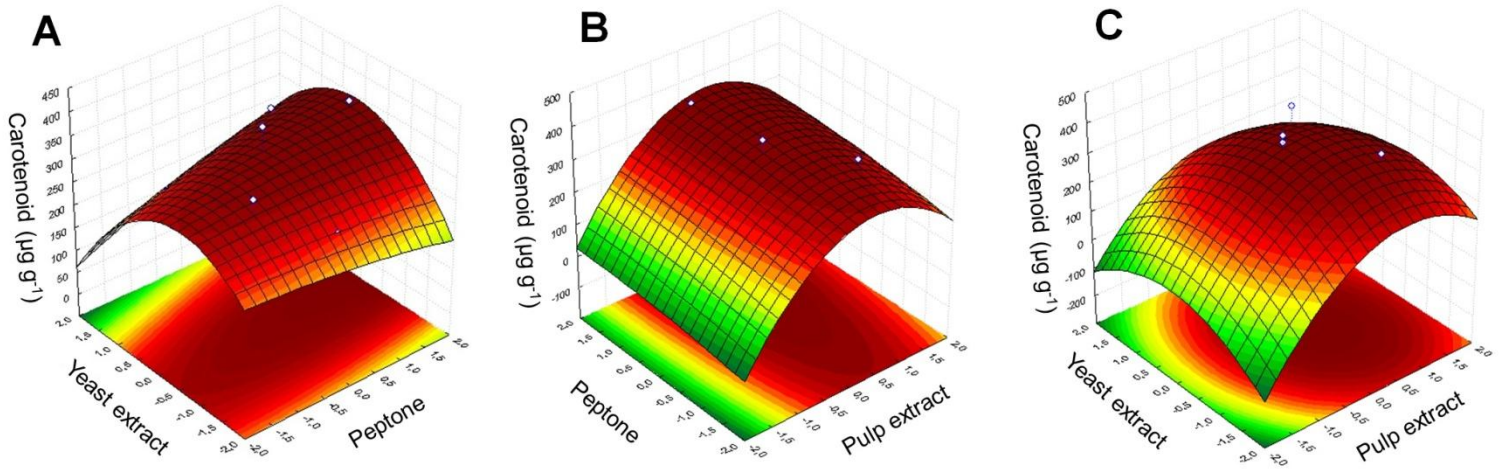

Figure 2 Surface plots of the responses in specific carotenoids production by *Rhodotorula mucilaginosa* CCMA 0156 in coffee pulp extract. (A) Specific carotenoids production as a function of yeast extract and peptone. (B) Specific carotenoids production as a function of concentration of coffee pulp extract and peptone. (C) Specific carotenoids production as a function of concentration of coffee pulp extract and yeast extract

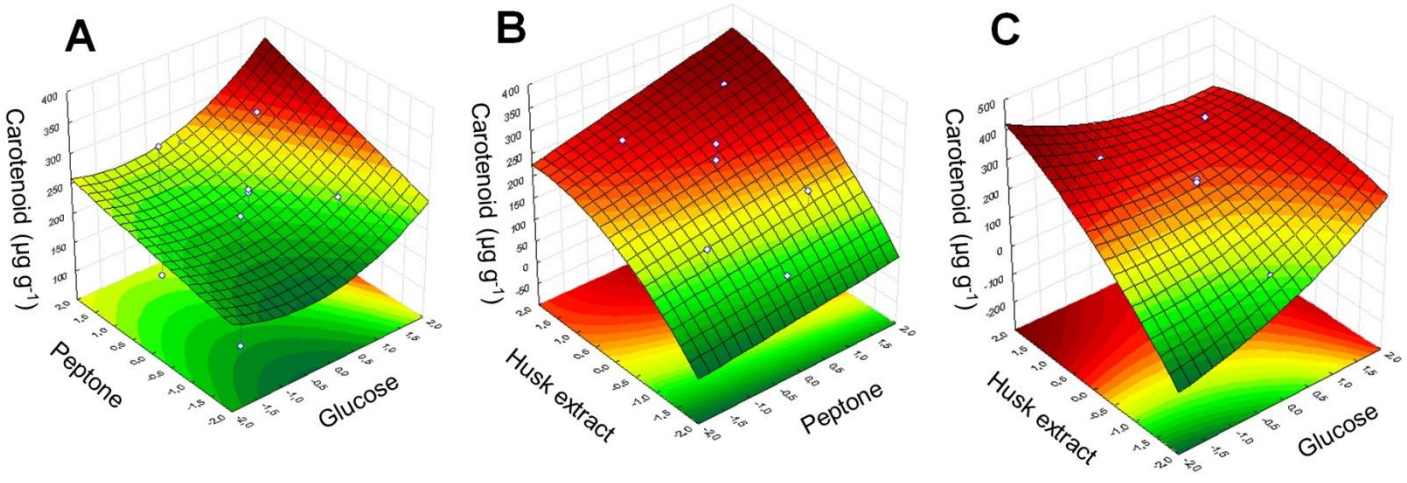

Figure 3 Surface plots of the responses in specific carotenoids production by *Rhodotorula mucilaginosa* CCMA 0156 in coffee husk extract. (A) Specific carotenoids production as a function of peptone and glucose. (B) Specific carotenoids production as a function of concentration of coffee husk extract and peptone. (C) Specific carotenoids production as a function of concentration of coffee husk extract and glucose

Validation of the experimental design

Validation in coffee pulp extract, the modified model predicted optimal values of the variables in the coded units of X_1 (pulp extract) = 0.84, X_2 (peptone) = 0.0 and X_3 (yeast extract) =1.68. The experiment in coffee husk extract, X_1 (glucose) = 1.68, X_2 (peptone) = 1.68, and X_3 (husk extract) =1.68, this values were obtained by solving the regression equation. Thus, the optimal conditions of the culture medium (PE) were as follows: pulp extract 6.68 %, peptone 10.04 g l^{-1}, and yeast extract 3 g l^{-1}. The model predicted that the production of carotenoids could reach a value of 347.54 µg g^{-1} (Eq. 1). The optimal conditions of the culture medium (HE) were as follows: glucose 6.36 g l^{-1}, peptone 3.68 g l^{-1}, and husk extract 8.36 %. The model predicted that the production of carotenoids could reach a value of 325.29 µg g^{-1} (Eq. 2). To confirm the model's adequacy in predicting the highest specific carotenoids yield, three additional experiments were performed using these optimized conditions. The mean production of specific carotenoids obtained from these validation experiments was 361.29 ± 36.0 µg g^{-1} (PE) and 296.58 ± 13.2 µg g^{-1} (HE), in agreement with the predicted values. The results verified the models and the optimal points.

Figure 4 shows the fermentation parameters monitored during the time carotenois production (0, 48 and 120 h) by *Rhodotorula mucilaginosa* CCMA 0156 in validation assays in pulp extract and coffee husk extract. The yeast had similar behavior in both medium, once the production of carotenoids was accompanied by increased of biomass and consumption reducing sugars. However, the production of biomass in the fermentation was relatively higher in husk extract (HE) (6.6 g l^{-1}) than pulp extract (PE) (4.5 g l^{-1}) 120 h of cultivation, whereas the concentration and consumption was greater in husk extract . This may have occurred because the consumption of reducing sugar HE

was higher than in PE coming to the reduction of 72.65% and 50%, respectively, at the end of fermentation. Consequently the production of total carotenoids was 3.2 x higher in HE (114.02 µg l^{-1}) than PE (44.39 µg l^{-1}) (Figure 4). The initial pH in both medium was about 4.5, remained constant in the first 48 h and then had a slight increase of about 5.6 (PE) and 6.7 (HE).

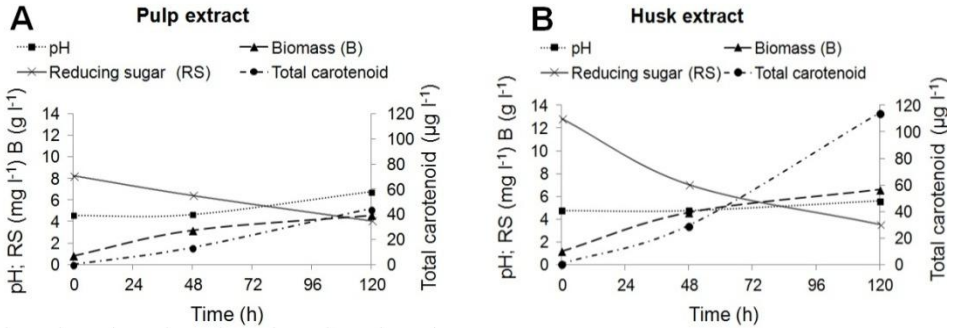

Figure 4 Fermentation profile of *Rhodotorula mucilaginosa* CCMA 0156 during the production of total carotenoids. A) Coffee Pulp extract and B) Coffee husk extract in the validation experiments on 120 h at 28 ° C, 160 rpm in the dark

Discussion

The coffee pulp and husk are the first by-products obtained during processing of the coffee fruit representing 29% and 12% of the dry weight of the all berry, respectively (Naidu and Murthy, 2012). These by-products have similar chemical composition. Coffee pulp and husk are rich in carbohydrates (32 - 35%), proteins (7 - 15%) and mineral (10.7%) (Brand et al, 2001; Ulloa-Rojas et al, 2003; Jimenez & Esquivel 2012). Although the chemical composition of the coffee processing waste vary with the coffee variety, growing conditions, processing mode, the developmental stage at which the grains were harvested (Pandey et al., 2000; Elias, 1979) most found values for coffee pulp and coffee husk was similar to other authors (Silva et al, 2013;. Esquivel and

Jiménez, 2012; Murthy and Naidu, 2012; Navia et al, 2011.). The presence of high amounts of sugars, proteins and cellulose indicate that these residues are very promising for use in many biotechnological processes (Bonilla-Hermosa et al., 2014).

The great commercial demand for natural carotenoids has drawn attention to the development of appropriate and inexpensive biotechnology techniques, including the use of industrial agro waste as sources of carbon and / or nitrogen (Asku and Eren., 2005; Taskin et al, 2011; Marova et al., 2012, Braunwald et al., 2013). In our study, the use of coffee by-products, pulp and husk, for obtaining extracts of a culture medium for the production of carotenoids by yeast and bacteria as an excellent alternative. Since these byproducts provided the carbon source necessary for the biosynthesis of carotenoids. As with other agro-industrial waste they are cheap and easily available produced and there use contributes to a reduction in production cost (Asku and Eren, 2005; Mata-Gómez et al., 2014).

Carotenoids yeast and bacteria accumulate in lipid particles in the cell and therefore can be extracted using various organic solvents (eg acetone, petroleum ether, hexane, ethyl ether, dichloromethane, methanol). The efficiency of the method varies depending on the characteristics of the microbial cells like the presence of a cell wall and the influence of the cell surface (Egyházi et al. 2004). However there is still no standard technique to ensure maximum efficiency of extraction for any microorganism (Park et al, 2007;. Valduga et al., 2009). The extraction usually requires an efficient disruption of the cell membrane. Acid treatments can be used in the lysis of the cell membrane. But can result in considerable loss of carotenoids (Egyházi et al. 2004). Chemical extraction is a possible substitute for mechanical disruption due to its simplicity, short team processing and relatively low cost (Fontana et al. 1996).

Other methods of extraction of intracellular carotenoid based on enzymatic reactions (Fang and Wang, 2002;. Sorebakken et al, 2004) and extraction with supercritical carbon dioxide have been reported (Lim et al, 2002;. Montero et al., 2005). Monks et al. (2012) evaluated both chemical methods, the enzymatic and cell disruption and ultrasound to obtain carotenoids produced by the yeast *Sporidiobolus salmonicolor* (CBS 2636). The maximum concentration of total carotenoids (2.875 g l^{-1}) was obtained by treatment with supercritical CO_2 (300 bar 120 min^{-1}) followed by dimethylsulfoxide to disrupt the cell, and extraction with a solution of acetone / methanol (7: 3 v/v). It was verified and Increase in system pressure led to an enhancement in mass transfer rate. The use of supercritical CO_2 combined with DMSO to disrupt microbial cells may be seen as a promising, attractive technique towards carotenoids recovery due to the low critical temperature and critical pressure mild exhibited by the solvent. Although the best result was verified with the use of SC-CO_2 to disrupt the lyophilized cells, the content of carotenoids obtained can be considered very low compared to content extracted from the microbial cells (Valduga et al., 2009a).

Recovery of carotenoids of cells *R. mucilaginosa* CCMA 0156, *R. mucilaginosa* CCMA 0340 and *Dermacoccus nishinomiyaensis* CCMA 0685 was tested using three different methods of extraction with chemical solvents. All solvent combinations were effective in the extraction of carotenoids, but with differences in performance of recovery. Studies report that mixtures of solvents are more efficient than a pure solvent on recovery of intracellular synergistic effect due to carotenoids (Park et al., 2007).

The production modes in the three fermentation and recovery of carotenoids extracted by the three methods were statistically significant. The differences observed in the carotenoid production of income are associated with each studied strain. According to Valduga et al. (2009) the differences in the

results of extraction may be attributed to (1) the ability of a given solvent to increase the cell membrane permeability and / or (2) the solubility of the carotenoid in a given solvent. Other factors may account for the differences in the recovery step (downstream) as the distinct used microorganism strains and their cell characteristics, the way of maceration of the cells and the degree of rupture with liquid nitrogen, inter alia (Park et al. 2007; Monks et al, 2012). The positive results reported here suggest that further study on the use of mixtures containing other solvents to isolate carotenoids pigments from microbial cells is necessary.

In our study, the maximum production of carotenoids *by Rhodotorula mucilaginosa* CCMA 0156 was observed during the stationary phase (data not shown) at 96 h of fermentation. In addition, the maximum yield was accompanied by maximal production of biomass, as reported in the literature. The accumulation of carotenoids in most yeast cells and bacteria starts in the late log phase and continues in the stationary phase (Buzzini et al., 2005; Libkind and Broock, 2006; Marzieh et al, 2009; Valduga et al, 2009). Valduga et al. (2011), studying yeast *Sporidiobolus salmonicolor* (CBS 2636), reported that carotenoid formation was associated with the end of exponential growth phase. The maximum total carotenoid production (3.426 µg l^{-1}) and biomass (11.2 g l^{-1}) were observed at 90 h of fermentation during the stationary phase.

The production of carotenoids by *R. mucilaginosa* CCMA 0156, *R. mucilaginosa* CCMA 0340 and *Dermacoccus nishinomiyaensis* CCMA 0685 was tested by two natural medium (pulp extract and coffee husk extract) without addition of nutrients to assess the ability of microorganisms to ferment carbon sources of the substrate and synthesize carotenoids. Simultaneously, a complex synthetic medium (YM) was used as control. The results are interesting, since natural and economic means, such as the extract of the pulp and husk extract can

be used in the production of carotenoids and replace the synthetic means in industrial processes.

The productivity of a bioprocess in a given system depends on the nutritional and physical conditions of culture, not only affecting cell growth and the production of pigment (Valduga et al., 2011). Thus, various kinds of microorganisms accumulate carotenoids in response to stress environmental conditions (Bhosale, 2004). Thus, control of physiological and nutritional stress can be used for increased production of pigment. However, the biotechnological synthesis of carotenoids is influenced by many factors involved in the processes that can affect the yields and operation costs (Mata-Gómez et al. 2014).

One of the most important factors to consider is the carbon source. Carbon source is the most studied parameter to influence carotenogenesis. Metabolism of yeasts acts depending on the kind of carbon source in the medium (Hannibal et al, 2000). Sucrose and glucose are carbon sources most commonly used in the production of carotenoids (Valduga et al., 2009). In our study, the synthetic YM medium was used as a control to compare the growth and accumulation of intracellular carotenoids among strains with natural means (coffee pulp extract and husk extract). *R. mucilaginosa* CCMA 0156 and *R. mucilaginosa* CCMA 0340 sugars consumed faster than bacteria in the three media, whereas the synthetic media consumption was almost total. The sugar present in synthetic medium (YM) is glucose it was easily consumed by the microorganisms. Already in the media with pulp extracts and coffee pods addition of glucose, other sugars such as sucrose, fructose, arabinose, galactose, maltose and polysaccharides could be present (ABIC, 2015), which may have slowed down the consumption of reducing sugars by the microorganisms.

In the production of carotenoids by *R. glutinis* using low cost substrates (glucose, sucrose, molasses and lactose from the whey), the highest concentration of carotenoids (125 mg l^{-1}) was obtained with 20 g l^{-1} sucrose

molasses (Asku and Eren, 2007). Marova et al. (2012) also used in agro-industrial waste as a substrate for the production of carotenoids and biomass by yeasts *Sporobolomyces roseus*, *Rhodotorula glutinis*, *Rhodotorula mucilaginosa*. Cheap residues can be used in the industrial production of biomass rich in carotenoids. The best production of biomass (45 g l^{-1}) and β-carotene (56 mg l^{-1}) was obtained in a medium containing whey, the cultivation of *R. glutinis*. According to the authors, biomass production and pigment differs according to the type of substrate used. As observed in our results, the production of biomass and carotenoids by *R. mucilaginosa* CCMA 0156, *R. mucilaginosa* CCMA 0340 and *Dermacoccus nishinomiyaensis* CCMA 0685 was different in each fermentation medium (YM, PE and HE). Since the synthetic medium, there were three different sources of nitrogen (3 g l^{-1} yeast extract, 3 g l^{-1} of malt extract and 5 g l^{-1} peptone) and 10 g l^{-1} glucose as a source of carbon. Already in natural medium 5% (PE and HE), carbohydrates as carbon source and protein as a nitrogen source.

The production of biomass and carotenoid by *Rhodotorula rubra* was studied, molasses and cane syrup by Banzatto et al. (2013), supplemented with two nitrogen sources. The intracellular carotenoid production was increased in all studied media without supplementation (0.329 mg g^{-1}). In the medium of the base of sugarcane juice without supplementation, there was a lower consumption of carbohydrates, whereas urea-based medium with supplementation had the highest consumption. Libkind and Broock (2006) studied growth and production of carotenoids in semi synthetic using ammonium sulfate and urea as nitrogen source, together with agro-industrial waste (molasses, corn syrup, malt extract) as carbon sources. The maximum production of the pigment 300 µg g^{-1} was produced by *Rhodotorula mucilaginosa* CRUB 0195.

In addition to the different sources of carbon and nitrogen which are associated with the microbial production of carotenoids, the pH is one of the

most important physical parameters for consideration. They have a huge influence on cell growth and product formation (Valduga et al., 2009). Frengova et al. (1994) disclosed that carotenoid biosynthesis naturally causes changes in pH of the culture medium, as a result of yeast growth with excretion compounds as acetic acid, alcohol or intermediate of the citric acid cycle during their adaptation phase, causing the drop in pH. In general, the pH of the medium decreases during the first 72 h production, followed by an increase during the intense phase carotenogenesis. Thereafter, the pH remains constant indicating the end of production (Frengova et al., 1994). Similarly, in our study, during the production of carotenoids by *Rhodotorula mucilaginosa* CCMA 0340, *R.mucilaginosa* CCMA 0156 and *Dermacoccus nishinomiyaensis* CCMA 0685 on synthetic medium (YM) the pH had the same behavior as reported in the literature.

However, during the cultivation of micro-organisms in coffee pulp extract and husk extract, the pH was different than reported in other studies, it remained constant until 48 h of fermentation. After this period all crops increased the pH around 7 until the end of fermentation. The coffee pulp extract and husk extract are more acidic than the synthetic medium (pH 6), whose pH is close to 5, this fact may explain why the pH remained stable in the media in the early hours of fermentation. The strong influence of pH in product and cell yield was reported by Hu et al. (2006). At initial pH 5.0, in a system without pH control, they obtained the lowest yields $Y_{X/S}$ (0.18 g g^{-1}) and $Y_{P/S}$ (0.24 mg g^{-1}). The highest yields obtained by those authors ($Y_{P/X}$ = 1.74 mg g^{-1} and $Y_{P/S}$ = 0.36 mg g^{-1}) were found with pH control set at 6.0 up to 80 h and then set to 4.0 up to the end of bio-production.

Valduga et al. (2009c) reported that the fermentation kinetics showed the maximum concentration of the total carotenoids was reached after 90 h of fermentation and carotenoid bio-production was partially associated with cell

growth. In our study, the maximum concentration of the total carotenoids was reached after 96 hours of fermentation. Carotenoid production started in the exponential growth phase and extended into the stationary phase. The increase in the specific yield of carotenoids is indicative of an increase in carotenoid biosynthesis in the yeast cells (Liu et al., 2006). Perhaps this behavior is related to changes in metabolism and cellular stress.

Rhodotorula mucilaginosa CCMA 0156 was the strain that showed the best results in the production of carotenoids using coffee pulp extract and husk extract compared with *R. mucilaginosa* CCMA 0340 and *Dermacoccus nishinomiyaensis* CCMA 0685. Thus, an experimental design based on the response surface methodology was developed for improving production in the coffee pulp and husk extracts.

Several factors as sources of carbon and nitrogen, minerals, pH, temperature, aeration, agitation and others that influence the production of carotenoids by yeasts have been studied in optimization studies (Malisorn and Suntornsuk, 2008; Ni et al., 2008; Valduga et al., 2009; Valduga et al., 2009c, Maldonade et al., 2012). In our study, the maximum production of specific carotenoids by *Rhodotorula mucilaginosa* CCMA 0156 in pulp extract (6.68 %) was 361.29 ± 36.0 µg g^{-1} obtained with 2 g l^{-1} glucose, 3 g l^{-1} yeast extract and 10.04 g l^{-1} peptone. Production in husk extract (8.36 %) was 296.58 ± 13.2 µg g^{-1} obtained with 6.36 g l^{-1} glucose and 3.68 g l^{-1} peptone. After the optimization process, carotenoid production in PE and HE increased 4.43-fold and 3.08-fold, respectively. These results showed that the statistical experimental design offers a practicable approach to the implementation of optimization of carotenoid production.

Our results were higher than the studies Maldonade et al (2012) the maximum carotenoid production by *R. mucilaginosa* (152 µg g^{-1}) was obtained with 5 g l^{-1} yeast extract and 10 g l^{-1} glucose. Probably the composition of the

coffee pulp extract and husk extract medium used in our study favored the growth and production of carotenoids by *R. mucilaginosa* CCMA 0156. The production of carotenoids by *R. mucilaginosa* CCMA 0156 was not influenced by malt extract (not significant $p > 0.05$), however, Valduga et al. (2009a) and Valduga et al. (2009c) study with *Sporidiobolus salmonicolor* (CBS 2636) reported that the maximum production of carotenoids by this yeast was dependent on the malt extract.

The production of carotenoids by *R. mucilaginosa* using agroindustrial substrates (sucrose lactose whey and molasses) was also studied by Aksu and Eren (2005). The authors reported that in general, the increase in sugar concentration increased the growth of yeast and the total carotenoids production (89 mg l^{-1}) was obtained when 20 g l^{-1} sucrose molasses was used the the carbon source. Similarly Aksu and Eren (2007) studied the carotenoid production by *Rhodotorula glutinis* concluding that an increase in sugar concentrations (initial glucose concentration 5 g l^{-1}) in the growth medium increased the growth of yeast and carotenoid production (69 mg l^{-1}).

Carotenoids production by fermentation can become industrially feasible if the cost of production can be minimized by use of cheap industrial by-products as nutrient sources. A number of researchers have investigated carotenoids production from various grains (oats, wheat, barley, corn, rice, rye), lipids and related substances, glycerol, cellobiose, sugar cane molasses, grape must, and cheese whey by different strains in shake flask fermentation (Bhosale and Gadre, 2001; Aksu and Eren, 2005; Valduga et al., 2008; Valduga et al., 2009b; Schneider et al., 2013).

Tinoi et al. (2005) demonstrated the effectiveness of using a widely available agro-industrial waste product as substrate and the importance of the sequential simplex optimization method in obtaining high carotenoid yields by *R. glutinis*. Under optimized conditions the cell dry weight was 10.35 ± 0.13 g l^{-1}

and the total carotenoid content was 3.48 ± 0.02 mg l^{-1} using 23.63 g l^{-1} of H_2SO_4-hydroyzed mung bean waste flour, 51.76 g l^{-1} of sweet potato extract, pH 5.91, 30.3 °C, agitation rate of 258 rpm and incubation time of 94.78 h. In our work possibility to use waste substrates and, moreover, to increase the biomass and pigment production in waste medium was confirmed.

The coffee processing residues proved potential by-products for use as culture media for the production of microbial carotenoids. Future work can be focused on the functional and structural characterization studies of these molecules can be utilized for possible industrial use.

Acknowledgements

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Conflict of Interest

Authors declare that they have no conflict of interest.

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ARTIGO 2

Antioxidant and antimicrobial activities of carotenoids produced by *Rhodotorula mucilaginosa* CCMA 0156 in submerged fermentation using coffee processing waste

Versão preliminar a ser submetida ao periódico *Applied Microbiology and Biotechnology*, preparada de acordo com suas normas de publicação

Antioxidant and antimicrobial activities of carotenoids produced by *Rhodotorula mucilaginosa* CCMA 0156 in submerged fermentation using coffee processing waste

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Abstract

This work aimed was to evaluate the antioxidant and antimicrobial activities of the carotenoids produced by *Rhodotorula mucilaginosa* CCMA 0156 in submerged fermentation using coffee processing waste. Furthermore, to quantify β-carotene by liquid chromatography (HPLC-DAD). Coffee pulp extract (6.68 %) and husk extract (8.36 %) were mediums used during fermentation, added by yeast extract (3 g l^{-1}), peptone (10.04 g l^{-1}) and glucose (2 g l^{-1}); peptone (3.68 g l^{-1}) and glucose (6.36 g l^{-1}), respectively. Both mediums contained Tween 80 (0.5 %). The production of carotenoids total by yeast in the pulp extract was 16.36 mg l^{-1} and husk extract was 21.35 mg l^{-1}. Carotenoids produced exhibited antioxidant and antimicrobial activities against pathogenic bacteria: *Salmonella cholerasus*, *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes* and toxigenic fungi: *Aspergillus flavus*, *A. parasiticus*, *A. carbonarius* and *A. ochaceus*. β-carotene represented 4.1 and 4.5% of the total carotenoids produced in coffee pulp extract and husk extract, respectively.

Keywords: Pigments; *Rhodotorula mucilaginosa*; β-carotene; Coffee pulp; Coffee husk; Antioxidant; Antimicrobial.

Introduction

Carotenoids are liposoluble pigments belonging to group of more than 600 molecules being most terpenes (C_{40}) and widely distributed in nature (Vachali et al., 2012). These pigments are exclusively synthesized by plants and microorganisms (Frengova and Beshkova, 2009; Walter and Strack, 2011) and are responsible for the wide variety of colors yellow, orange and red found in nature (Vachali et al, 2012).

Carotenoids protect cells against photooxidative damage and therefore present important applications in, disease control, environment, food and nutrition and as potent antimicrobial agents (Kirti et al., 2014). The interest in carotenoids has increased in recent years due to its proven pro-vitamin A activity and evidence of other biological properties such as activities antioxidant, anticarcinogenic, macular degeneration and prevention of cardiovascular

diseases (Krinsky and Johnson, 2005; Maldonade et al., 2008; Mata-Gómez et al., 2014). Moreover, natural colorants market has shown very promising because of the tendency of non-use of artificial additives in food (Squina and Mercadante, 2003; Kayser et al., 2007). In recent years there has been growing interest in microbial production of carotenoids, as well as being a natural source, can be obtained at short time and at any time of year (Valduga et al., 2011).

Currently, the commercial use of pigments produced by microorganisms is limited by the high cost of production. However, the use of low-cost agro-industrial waste as a source of nutrients in the fermentation and optimization of process conditions can be an alternative for lower cost (Aksu and Eren, 2005; Maldonade et al., 2012; Marova et al., 2012). The types and amounts of carotenoids produced may vary depending on the carbon and nitrogen sources, minerals, vitamins, pH, aeration, temperature, light and stress (Aksu and Eren, 2005; Valduga et al., 2009a). The production of carotenoids from different substrates has been reported in some papers with grains (oats, wheat, barley, maize, rice, rye), lipids, glycerol, cellobiose, sugar cane, molasses, grape juice, cheese and whey milk from different microbial strains (Bhosale and Gadre, 2001; Aksu and Eren, 2005; Valduga et al., 2008; Valduga et al., 2009a; Maldova et al., 2012; Schneider et al., 2013).

In this context, the study aimed to evaluate the antioxidant and antimicrobial activities of the carotenoids produced by *Rhodotorula mucilaginosa* CCMA 0156 in submerged fermentation using coffee processing waste. Furthermore, to quantify β-carotene by liquid chromatography (HPLC-DAD).

Materials and methods

By-products and Pretreatment

Coffee husk and pulp from dry and semi-dry process respectively of the coffee beans (*Coffea arabica* L., variety red Catuaí 99) was supplied by a coffee-producing unit located in the southern area of the state of Minas Gerais (Brazil). When obtained, the materials were immediately frozen at -20°C. The coffee husk and pulp was dried at 65°C until it reached a constant weight (AOAC) (1995). Later, were ground in a Willey mill (1.0 mm) and stored in plastic flasks. The substrates were pretreated individually with 0.06 % (w/v) KOH. Following this, 50 g of each substrate dry and milled was resuspended in 1 l of distilled water containing KOH and sterilization was carried out at 121 º C for 30 min (Machado et al., 2002). The extracts were filtered with Whatman Nº. 1 and used for fermentation studies.

Micro-organism and fermentation conditions

The strain used in this study is belonging to the Culture Collection of Agricultural Microbiology (CCMA) of Department of Biology, Federal University of Lavras, Brazil. *Rhodotorula mucilaginosa* CCMA 0156 (isolated coffee fermentation). Initially culture were reactivated in 1 ml medium (YM) (Yeast Malt Extract) (g l^{-1}) (w/v): 3.0 yeast extract, 3.0 malt extract, 5.0 peptone and 10.0 glucose, 28 ° C for 48 h. Added in 9 ml tube of the same medium for 24 h, after was transferred to 250 ml Erlenmeyer flasks with 90 ml of the same medium and incubated for 48 h. Before inoculation the fermentation media, the cells were centrifuged at 9000 rpm, 4 ° C for 10 min and washed 2 x with distilled water. The starter culture was 10.0% (v/v) inoculated by batch

fermentation. The production of carotenoids was performed in two different medium, containing coffee pulp extract (6.68 %), peptone (10.04 g l^{-1}), yeast extract (3 g l^{-1}), glucose (2 g l^{-1}) and tween 80 (0.5 %). And another containing husk extract (8.36 %), glucose (6.36 g l^{-1}), peptone (3.68 g l^{-1}) and and tween 80 (0.5 %). The cultivations were carried in a shaker under temperature control and agitation in 500 ml Erlenmeyer flasks containing 300ml of medium, inoculated with 10^7 cél ml^{-1} and incubated at 28 ° C, 160 rpm for 5 days in the dark. The experiments were conducted in triplicate for each medium.

Recovery and determination of carotenoids

The recovery of total carotenoids was carried out according to the methodology described by Valduga et al. (2009), with modification. The carotenoids produced were recovered by acetone: methanol (7: 3, v / v). Previously biomass recovered from the fermentation medium was centrifuged (9,000 × g, 4 ° C, 10 min) and dried in oven at 65 ° C for 24 h. Then macerated in liquid N_2 and added to 2 ml of dimethylsulfoxide (DMSO) and heated in a water bath at 55 ° C / 30 min. After this time, 2 ml of mixed solvent were added and centrifuged (5,000 × g, 4 ° C, 10 min). The supernatant was separated, and successive extractions were carried out until both solvent and cells remain colorless. The solvent was evaporated in a nitrogen gas atmosphere and the pigments were solubilized in 5 ml methanol.

The absorbance of the sample after the extraction was measured in a spectrophotometer (Biospectro, Model SP-220). The concentration of total carotenoids was estimated by the maximum absorbance at 450 nm, using the equation described by Davies (1976). The coefficient of absorbance used was that referent to β-carotene: $E^{1\%}_{1\ cm}$ = 2550, for methanol (Silva et al., 2004). Concentration of carotenoids was expressed in terms of total carotenoids (μg l^{-1})

and specific production of carotenoids (μg g^{-1}). The specific production of carotenoids represents the total concentration of carotenoids (μg) in relation to the biomass of dried yeast obtained in 1 l of a fermented medium (Davies 1976).

Determination of Biomass

After extraction of carotenoids, the cells were washed with distilled water and centrifuged at 5,000×g for 10 min at 4°C. The biomass was quantified through drying at 65°C until a constant mass.

Determination of reducing sugars, pH and cell growth

The procedure used for quantification of glucose was the DNS (3,5-dinitrosalicylic) methodology as described by Miller (1959) and the staining intensity was measured by spectrophotometer (Biospectro, Model SP-220) at a wavelength of 540 nm. The pH of the culture media was determined using a digital pH meter (Digimed analytical, Model DM22). Cell growth was evaluated by counting in Neubauer Chamber.

Determination of sugars and acids produced during fermentation

The carbohydrate (glucose, sucrose and fructose) and organic acid (acetic acid, lactic acid, malic acid, citric acid and succinic acid) analyses were carried out using a liquid chromatography system (Shimadzu, model LC-10Ai, Shimadzu Corp., Japan) equipped with a dual detection system consisting of a UV–Vis detector (SPD 10Ai) and a refractive index detector (RID-10Ai) (Duarte et al., 2009). A Shimadzu ion exclusion column (Shim-pack SCR-101H, 7.9 mm × 30 cm) was operated at 30 °C for carbohydrates and 50 °C for acids.

Perchloric acid (100 mM) was used as the eluent at a flow rate of 0.6 ml min^{-1}. The acids were detected via UV absorbance (210 nm), while the alcohols and carbohydrates were detected via RID. All samples were analyzed in triplicate, and individual compounds were identified based on the retention time of standards injected using the same conditions. The sample concentrations were determined using an external calibration method. Calibration curves were constructed by injecting different concentrations of the standards under the same conditions of sample analysis and the areas obtained were plotted a linear curve whose equation was used to estimate the concentration of the compounds in the sample.

Quantification of β-carotene

The quantification of β-carotene was performed only extracts of carotenoids obtained from the means of validation tests: pulp extract and husk extract. Chromatographic analyses were conducted by Analysis Center and Prospecting Chemistry (CAPQ) of the Chemistry Department (DQI) of the Federal University of Lavras. The β-carotene content was determined according to procedures described by Maldonade et al. (2008), adapted. The extracts were concentrated in nitrogen gas, redissolved in 1 ml methanol and filtered through a nylon membrane filter 0.22 μm, (Millipore) before injection. Analyses were performed on a liquid chromatograph of high efficiency – HPLC (Shimadzu, Japan), equipped with quaternary pump LC-20AT model, diode array detector (DAD) EPDM-20A model, degasser DGU-20A5 model, interface CBM-20A model and automatic gun with autosampler SIL-20A model. The separations were carried out using A VP-ODS Shim-pack C18 column 25 cm x 4.6mm x 5μm and Shim-pack guard column GVP-ODS C18 column 10mm x 4.6mm x 5μm. The mobile phase was tetrahydrofuran: H_2O: methanol from 15:4:81

(v/v/v) to 30:0:70 (v/v/v) in a linear gradient in 55 min, maintaining this proportion until the end of the run. The flow rate was 0.6 ml min^{-1}. The column thermostat was set at 40°C, the injection volume was 20 µl and analyzed at 450 nm. The retention time of β-carotene extracted from *Rhodotorula mucilaginosa* CCMA 0156 was 32.487 min and quantified against peak area calibrations calculated from the standard curves of the β-carotene (1 mg ml^{-1}) standard (Sigma, USA).

Antioxidant activity

DPPH radical

The antioxidant activity was determined by measuring the radical scavenging activity of DPPH (1,1-diphenyl-2 picrilidrazil) according to the methodology described by Brand-Williams and Berset (1995), with adaptation. Aliquots of the extracts of the total carotenoids (0.25 ml) were placed in test tubes and added to 2.75 ml of the DPPH solution in methanol (0.06 mM). The tubes were left at rest in dark. At the end of 15, 30, 45 and 60 minutes, the absorbance of the tubes was measured at 515 nm. The ability to sequester radical was expressed as a percentage and calculated relative to the control (no antioxidant), using the expression described by Miliauskas et al. (2004) (Equation 1).

$$\%\,\boldsymbol{sequestro} = \frac{\boldsymbol{Abs\ controle - Abs\ amostra}}{\boldsymbol{Abs\ controle}} \times \mathbf{100} \qquad (1)$$

For comparison, synthetic antioxidant: butylated hydroxytoluene - BHT (100 ppm), ascorbic acid - AA (50 ppm) and β-carotene (100 ppm) were used as standard. Analyses were performed in triplicate.

Co-oxidation system β-carotene / Linoleic Acid

Oxidation inhibition power was evaluated by the discoloration of the β-carotene/linoleic acid system as described by Marco (1968) and modified by Miller (1971). Fifty µl aliquot of β-carotene solution (20 mg ml^{-1} in chloroform) was placed in a 250 ml Erlenmeyer flask with 40 µl of linoleic acid, 1 ml chloroform, and 530 µl of Tween 40. After homogenization, the chloroform was completely evaporated with nitrogen. Deionised water (previously submitted to oxygen atmosphere for 30 minutes) was then added until formation of a clear emulsion with absorbance ranging from 0.6 to 0.7 at 470 nm. After the initial reading, the samples were kept at 40 °C in a water bath and the absorbance was measured after 2 hours. The decrease in absorbance was compared to the control (without antioxidant). Butyl hydroxytoluene - BHT (100 ppm), ascorbic acid - A (50 ppm) and Trolox (200 mg l^{-1}), synthetic antioxidants were used as positive control. The antioxidant activity was expressed as oxidation inhibition percentage, in relation to the control, according to the following Equation 2:

$$\textbf{Inhibition}\ \% = 100 - \left[\frac{(Ai - Af)}{(Ci - Cf)} \times 100\right] \quad (2)$$

Ai = extract initial absorbance; Af = extract final absorbance; Ci = control initial absorbance and Cf = control final absorbance.

Antibacterial activity

The antibacterial activity of the carotenoids was performed according to the method described by Gudiña et al. (2010) adapted. The evaluation of minimum inhibitory concentration (MIC) of carotenoids against pathogenic strains *Salmonella cholerasus* ATCC 6539, *Staphylococcus aureus* ATCC 13565, *Pseudomonas aeruginosa* ATCC 15442, *Listeria monocytogenes* ATCC 19117 and *Escherichia coli* ATCC 11229 was determined by the microdilution method in flat-bottom 96-well polystyrene microplates.

Previously strains were reactivated in BHI broth (brain heart infusion) (Merck) incubated at 37 ° C for 24 h. They were then peak at the same medium plates and incubated under the same conditions. Suspensions of bacteria were prepared in saline solution (NaCl 0.85%), the standard nephelometric McFarland scale of 1 ($3x10^8$ UFC ml^{-1}). The following concentrations of extracts carotenoids were prepared: 0.00, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50 % (v/v) in BHI broth in a final volume of 150 µl per well. The bacterial inoculum (8 log CFU ml^{-1}) was added to the wells at a volume of 10 µl. For all concentrations, wells were prepared without the bacterial inoculums, containing only extracts carotenoids diluted in the culture medium (BHI broth). Two wells not contain extract carotenoids and served as negative and growth controls. Synthetic β-carotene (Sigma) at 1 mg ml^{-1} was used as a positive control. Microplates were covered and incubated at 37 °C for 24h. Triplicate assays were performed for all the carotenoids concentrations for each strain. The absorbance was measured at 600 nm in microplate reader (Multiskan GO - Thermo Scientific) prior to the incubation (time zero) and after 24 h. From the absorbance value obtained at 24 h was subtracted the reading of the time zero, discounting from the same ones the absorbance concerning the culture medium and extracts carotenoids. The

growth inhibition percentages at different extracts of carotenoids concentrations for each micro-organism were calculated as:

$$\% \text{ Growth inhibition} = \left[1 - \left(\frac{Ac}{Ao}\right)\right] \times 100 \quad (3)$$

where A_c represents the absorbance of the well with a carotenoid concentration c and A_0 the absorbance of the control well (without carotenoid).

The minimum inhibitory concentration (MIC) was determined for each strain as the lowest concentration of carotenoid that completely inhibits measurable growth (A_{600} = 0). To establish the minimum bactericidal concentration (MBC), 10 µl of each well with no visible growth were inoculated on plates containing TSA medium (tryptone soya agar) (Himedia) incubated at 37 ° C for 24 h. The lowest concentration of carotenoids that did not allow growth was considered as the MBC for that strain. Plates that showed growth were considered to come from wells with bacteriostatic concentration of carotenoid.

Antifungal activity

The antifungal potential of the carotenoids extracts was evaluated according to the methodology proposed by Lopes et al (2013), with adaptations. Two strains of filamentous fungi producers aflatoxin - *Aspergillus flavus* (CCDCA 10520) and *A. parasiticus* (CCDCA 10510) and two producers ochratoxin - *A. ochaceus* (CCDCA 10612) and *A. carbonarius* (CCDCA 10608) were tested.

The fungi were grown for 7 days on plates containing MEA medium (Malt Extract Agar) g l^{-1}: 20 g of malt extract, 1 g of bacteriological peptone, 10

g glucose and 20 g agar). After, the solution was added tween 80 (0.1%) over the entire surface of the plate and held with the rasp handle Drigalski, thus obtaining a suspension of spores. The concentration of 10^5 spores ml^{-1} (30 µl) were added in 70 µl extract and incubated for 2 h at 28 °C. After this period, 10 µl was inoculated (microdrop) in the MEA. Plates were incubated for 7 days at 28 °C. For each fungi negative controls was performed, in the absence of carotenoid. Synthetic β-carotene (Sigma) at a concentration of 1 mg ml^{-1} was used as a positive control. The experiment was performed in triplicate.

Results and Discussion

The data of total and specific production of carotenoids, as well as biomass *Rhodotorula mucilaginosa* CCMA 0156 are shown in Table 1. Most production of carotenoid and biomass was in the medium with husk extract.

Tabela 1 Concentration of carotenoids (specific and total carotenoids) and biomass obtained by *Rhodotorula mucilaginosa* CCMA 0156 in different mediums (coffee pulp extract – PE and coffee husk extract – HE) after 120h fermentation

Medium	Specific carotenoids ($\mu g\ g^{-1}$)	Total carotenoids ($\mu g\ l^{-1}$)	Biomass ($g\ l^{-1}$)
PE	2065.94±42.0	16362.27±73.0	7.92±0.8
HE	2457.81±31.6	21358.40±67.2	8.69±0.3

Yeast has similar behavior in both fermentation media. The production of carotenoids was accompanied by a consumption of reducing sugars present in the fermentation medium, growth (log cel ml^{-1}) and increase in pH (Figure 1). Although the initial reducing sugar content in husk extract (HE) is greater than

in the pulp extract (PE), *Rhodotorula mucilaginosa* CCMA 0156 the percent consumption of sugars near 86.8% and 84%, respectively.

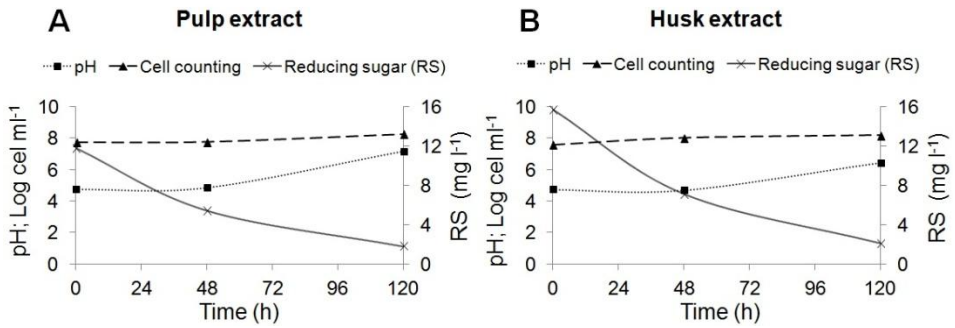

Figure 1 Population growth, consumption of reducing sugars and pH evolution in coffee pulp extract (A) and husk extract (B) during the production of carotenoids by *Rhodotorula mucilaginosa* CCMA 0156, at 28 °C, 160 rpm in the dark for 120 h

The contents of carbohydrates and acids of the fermentation media with 0, 48 and 120 h were analyzed by HPLC. The content of acids was evaluated in order to find out if the yeast used the presents sugars in the medium only to produce pigments, or have been converted into some other product of fermentation. *R. mucilaginosa* CCMA 0156 preferentially consumed glucose present in the mediums coffee pulp extract and husk extract in the first 48 h of fermentation. After this period, the yeast started use also fructose in both mediums. At the end of 120 h, all glucose and fructose was consumed. With respect to acids, only malic acid and succinic acid were found in small amounts (<0.15 g l^{-1}) during fermentation (data not shown).

Quantification of β-carotene

The quantification of β-carotene, carotenoids extracts of different medium coffee pulp extract and husk extract production are shown in the Table 2. The results of HPLC showed that the increased production of β-carotene was higher in coffee husk extract than in the pulp extract. The production of β-carotene in the coffee husk extract (HE), which corresponds of around 4.57 % of total carotenoids of the sample.

Table 2 β-carotene values in the carotenoids extracts produced by *Rhodotorula mucilaginosa* CCMA 0156 in different fermentation media (pulp extract - PE and husk extract - HE) and the relative percentage of this carotenoid with carotenoids

Medium	Total carotenoids ($\mu g\ l^{-1}$)	β-carotene ($\mu g\ l^{-1}$)	β-carotene %
PE	16362,27±73.0	670,52±20.6	4.10
HE	21358,40±67.2	975,60±51.8	4.57

In our study the difference in the production of β-carotene on different substrates may be related to composition of the coffee pulp extract and husk extract and nutrients added in each medium. The β-carotene production percentage was higher in husk extract supplemented with glucose (6.36 g l^{-1}) and peptone (3.68 g l^{-1}). According to our study, Bhosale and Gadre (2001b) reported that higher β-carotene content was produced by mutant of *Rhodotorula glutinis* grown in media containing ammonium nitrate, soy peptone or peptone the nitrogen sources. On the other hand, cultivation of *Rhodotorula glutinis* var. *glutinis* in medium containing ammonium sulphate resulted in production of highest cellular and volumetric carotenoids. Ammonium nitrate resulted in

highest percentage of β carotene, while peptone gave the highest percentage of torulene (El-Banna et al., 2012).

In relation the carbon source, El-Banna et al. (2012) reported that the color of pellets of the culture grown in media containing sucrose and glucose syrup had red hues due to the high percentages of torulene and torularhodin, respectively. While, fructose corn syrup and glucose gave pellets with yellow-red hues due to the high percentage of β-carotene. Bhosale and Gadre (2001b) found that the major carotenoid components produced by a mutant of *Rhodotorula glutinis* when glucose, fructose or sucrose were used as sole carbon source in the medium were β-carotene (69%), torulene (63%) or (60%), respectively.

The production of β-carotene by *R. mucilaginosa* CCMA 0156 in all medium used in this study was higher than carotenoid production by 4 strains *R. mucilaginosa* of reported Maldonade et al (2008). This authors studied the composition of carotenoids produced by Brazilian yeast strains, showing that the strains genus *Rhodotorula mucilaginosa*-12, *Rhodotorula mucilaginosa*-137, *Rhodotorula mucilaginosa*-108 and *Rhodotorula mucilaginosa*-135 produced 105 µg l^{-1}, 139 µg l^{-1}, 129 µg l^{-1} and 95 µg l^{-1} of β-carotene, respectively. However the proportion of β-carotene with respect to total carotenoids was similar to our work, around 20%. In contrast, *R. mucilaginosa* CCY 20-7-31 grown on potato medium and 5% salt showed a production of β-carotene 56 mg l^{-1} (Marova et al.,2012).

The ability of *R. mucilaginosa* yeast for growing on a variety of carbon sources, such as glucose, pulp extract, and husk extract is a remarkable advantage. When compared with the results obtained with other yeasts in the literature, the high carotenoid productivity of the yeast also suggests a feasible process (Asku and Eren, 2005; Malisorn and Suntornsuk, 2008; Marova et al.,2012; Banzatto et al., 2013). Thus, the yeast *R. mucilaginosa* will be one of

the most promising microorganisms for the commercial production of carotenoids by the use of agricultural wastes as a cheap carbon source.

Antioxidant activity

Analysis of antioxidant activity using the radical DPPH and assay using the co oxidation of β-carotene against linoleic acid have long been used to verify the scavenging capacity of free radicals in many natural products (Li et al., 2011). In Table 3, shows the results of the evaluation of the antioxidant activity of the carotenoid extracts produced by *Rhodotorula mucilaginosa* CCMA 0156.

Tabela 3 Antioxidant activity of carotenoids extracts produced by *Rhodotorula mucilaginosa* CCMA 0156 from the different culture media (coffee pulp extract – PE and husk extract - HE) under different methods (β-carotene / linoleic acid) and (DPPH)

Sample	% Proteção β-caroteno/ácido linoleico	% sequestro DPPH
PE	98.26±1.2	72.94±2.7
HE	64.13±5.2	76.74±1.2
TROLOX	73.70±1.4	-
BHT	85.92±2.9	90.82±1.2
AA	70.63±1.48	96.14±0.8
β-carotene	-	52.24±2.5

PE – pulp extract; HE – husk extract. Sinthetic antioxidants: Trolox - (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) BHT – Butylated hydroxytoluene; AA – ascorbic acid and β-carotene. DPPH – 1,1 – diphenyl - 2 - picrilidrazil

Comparing different detection methods the antioxidant activity, the cooxidação the β-carotene against linoleic acid was more effective in the analysis of carotenoids produced by the yeast extracts of than DPPH method.

Once carotenoids are composed of lipophilic nature, they have a greater affinity for this method (Kuskoski et al., 2005). Carotenoids produced in the media with pulp extract showed the best protection value 98.26%.These values were similar to those found for BHT (85.9%), lipophilic synthetic antioxidant and higher than Trolox and AA (hydrophilic), 73.7 and 70.6%, respectively.

The chronic diseases such as cancer, diabetes, cardiovascular and autoimmune disorders are known to associate with free radicals (Vachali et al. 2012). Microbial pigments like carotenoid, naphthaquinone and violacein have been shown to have a potent antioxidant activity due to their biological functions (Duran et al. 2012; Malik et al., 2012). Carotenoids could reduce the penetration of singlet oxygen by decreasing membrane fluidity (Vachali et al., 2012). Recent report by Kamila et al. (2008) showed that polar carotenoids such as zeaxanthin could mediate transmembrane proton transfer *in vivo*.

The common structural feature of carotenoid polyene chain is a long conjugated double bond system that forms the "backbone" of the molecule and influences their chemical, physical and biochemical properties. This chain may have cyclic end groups, which have substituents containing oxygen. The conjugated polyene and rich system of electrons is responsible for the antioxidant activity of carotenoids: both absorption as singlet oxygen free radicals to stop chain reactions in which they are engaged (Mcnulty, et al., 2007; Sikora et al., 2008). The presence of these connections also facilitates the oxidation of carotenoids, which causes a loss of color in food. It is because they are easily oxidized, which have antioxidant activity. The antioxidant efficiency as varies between different carotenoids, lycopene being regarded as the most efficient capturing singlet oxygen acts as a very potent antioxidant (Damodaran et al., 2008).

Antimicrobial activity

The antimicrobial activity of the total carotenoids isolated from *Rhodotorula mucilaginosa* CCMA 0156 was determined by measuring the growth inhibition percentages obtained for some pathogenic bacteria (Table 4). From those results, the MIC for each micro-organism was determined. Furthermore, whenever possible, the MBC was also determined. All total carotenoids were effective against all the microorganisms tested, although to different degrees.

Table 4 Percentage inhibition of growth (MIC) of pathogenic bacteria by the action of cartenoides extracts recovered from *Rhodotorula mucilaginosa* CCMA 0156 in different fermentation medium (coffee pulp extract – PE and husk extract - HE). Results are expressed as means ± standard deviations of values obtained from triplicate experiment

Micro-organism	**PE**				
	3.12 (0.076 μg μl^{-1})	6.25 (0.153 μg μl^{-1})	12.5 (0.306 μg μl^{-1})	25.0 (0.612 μg μl^{-1})	50.0 (1.225 μg μl^{-1})
Pseudomonas	40.0 ± 0.3	58.3 ± 0.5	65.6 ± 0.4	75.9 ± 0.5	100.0 ± 0.0*
Salmonella	66.4 ± 0.5	86.4 ± 0.4	95.6 ± 0.3	100.0 ± 0.0*	100.0 ± 0.0*
E.coli	47.1 ± 0.4	60.8 ± 0.3	66.1 ± 0.5	100.0 ± 0.0*	100.0 ± 0.0*
S.aureus	66.0 ± 0.3	71.5 ± 0.3	75.3 ± 0.4	100.0 ± 0.0*	100.0 ± 0.0*
Listeria	58.0 ± 0.5	69.7 ± 0.5	85.2 ± 0.09	100.0 ± 0.0*	100.0 ± 0.0*
Micro-organism	**HE**				
	3.12 (0.100 μg μl^{-1})	6.25 (0.200 μg μl^{-1})	12.5 (0.400 μg μl^{-1})	25.0 (0.800 μg μl^{-1})	50.0 (1.600 μg μl^{-1})
Pseudomonas	56.3 ± 0.5	65.3 ± 0.5	77.3 ± 0.5	89.9 ± 0.3	100.0 ± 0.0*
Salmonella	43.8 ± 0.3	54.1 ± 0.4	63.2 ± 0.6	100.0 ± 0.0*	100.0 ± 0.0*
E.coli	38.0 ± 0.7	42.4 ± 0.6	58.7 ± 0.5	73.2 ± 0.2	100.0 ± 0.0*
S.aureus	39.4 ± 0.5	52.9 ± 0.07	63.1 ± 0.3	88.1 ± 0.1	100.0 ± 0.0*
Listeria	44.5 ± 0.6	53.2 ± 0.2	75.1 ± 0.5	100.0 ± 0.0*	100.0 ± 0.0*

*Indicates the minimum bactericidal concentration (MBC)

For all the micro-organisms studied, the antimicrobial activity was observed even at low carotenoid concentrations, and a complete growth inhibition was achieved for the 4 micro-organisms at the highest carotenoid concentration assayed (50 v/v) - 1.225 µg μl^{-1} (PE) and 1.600 µg μl^{-1} (HE). *Salmonella*, *E.coli*, *Staphylococcus aureus* and *Listeria* exhibit the complete inhibition of growth by the action of carotenoid extract through PE concentrations (MIC) between 25-50 (v/v) (0.612 – 1.225 µg μl^{-1}). Furthermore, it is noticeable that even when MIC and MBC does not occurred at the same time, a high growth inhibition was observed from 73.2 to 89.9 % (HE), with the highest carotenoid concentration assayed (25 v/v - 0.800 µg μl^{-1}). Synthetic β-carotene (1000 µg ml^{-1}) used as positive control, was able to inhibit the growth of most bacteria tested, but had no action on the growth of *Listeria monocytogenes* ATCC 19117.

The majority of the studies in the literature on the antimicrobial potential of pigments are related to bacterial pigments or filamentous fungi. Lopes et al. (2013) evaluated the antimicrobial activity, the metabolite of MIC produced by *Penicillium chrysogenum* IFL1. Predominantly inhibition occurred against bacteria, *Staphylococcus aureus* (25 %), *Pseudomonas aeruginosa* (50 %) e *Bacillus cereus* (25 %) and phytopatogenic fungi *Fusarium oxysporum* (50 %). Endophytic fungal pigment was found to be superior to the commercial antibiotic Streptomycin against human pathogenic bacteria, *Staphylococcus aureus, Klebsiella pneumoniae, Salmonella typhi* and *Vibrio cholera* (Visalakchi and Muthumary 2010).

In our study, a low MIC value between (0.612 – 1.225 µg μl^{-1}) coffee pulp extract and (0.800 – 1.600 µg μl^{-1}) husk extract, was able to inhibit the growth of pathogenic bacteria tested. These values were lower than that reported by Kim et al. (2006) evaluated the antimicrobial potential of amino acid derivatives of *Monascus* pigments against several strains of bacteria and fungi.

The control red pigment exhibited minimal inhibitory concentration (MIC) values higher than 32 mg ml^{-1}. The authors report that the type of pigment is related to a specific mechanism of action in the target cell. Incubation of the L-Phe derivative with *Bacillus subtilis* caused cells to aggregate with formation of pellets. Whereas addition of monascus pigment derivatives decreased the oxygen uptake rate of *E. coli* in culture.

Future studies on mechanisms of action of carotenoids extracts produced by *Rhodotorula mucilaginosa* CCMA 0156 are necessary to understand the antimicrobial mode of action of these pigments.

Interesting preliminary results were observed regarding the antifungal potential of crude extracts of carotenoids. All extracts tested were able to completely inhibit the growth of all strains of *Aspergillus* evaluated. The results suggest that these carotenoids could be used to control the producer mycotoxin fungi in food. However, future studies should be performed to determine the minimum inhibitory concentration (MIC) of these extracts against these fungi as well as testing efficiency of these carotenoids other species of pathogenic fungi.

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Conflict of Interest

Authors declare that they have no conflict of interest.

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