Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.jzus.zju.edu.cn; www.springerlink.com
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Analysis of nicotine-induced metabolic changes in *Blakeslea trispora* by GC-MS*

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https://doi.org/10.1631/jzus.B1900459
Published online Feb. 5, 2020

Blakeslea trispora is a natural source of carotenoids, including β-carotene and lycopene, which have industrial applications. Therefore, classical selective breeding techniques have been applied to generate strains with increased productivity, and microencapsulated β-carotene preparation has been used in food industry (Li et al., 2019). In *B. trispora*, lycopene is synthesized via the mevalonate pathway (Venkateshwaran et al., 2015). Lycopene cyclase, which is one of the key enzymes in this pathway, is a bifunctional enzyme that can catalyze the cyclization of lycopene to produce β-carotene and exhibit phytoene synthase activity (He et al., 2017).

Nicotine is a cytotoxic natural alkaloid. A previous study revealed that nicotine adversely affects the carotenoid synthesis pathway of *Dunaliella salina* resulting in the accumulation of carotenoid intermediates and changes to carotenoid compositions (Fazeli et al., 2009). More importantly, nicotine is considered to be one of the most effective inhibitors of lycopene fermentation in *B. trispora*. By inhibiting lycopene cyclase, nicotine induces the cellular accumulation of lycopene and decreases γ-carotene production (Liang et al., 2016).

To efficiently manipulate the fermentation of lycopene in *B. trispora*, especially in the presence of inhibitors, the metabolic responses induced by nicotine throughout the fermentation process must be thoroughly characterized. The intracellular small molecule metabolites are affected by the combination of media, and the changes to metabolites may be due to the relationship between biological systems and environmental variations (Jia et al., 2016).

We examined the utility of a nicotine detection method involving gas chromatography-mass spectrometry (GC-MS) (Ṣpaiuc et al., 2014) for the highly sensitive and high-resolution detection of specific compounds (Villas-Bôas et al., 2003; Wagner et al., 2003). According to the high throughput detection of bioactive components (Zhang et al., 2019), we applied GC-MS to detect the variations in metabolites during the fermentation of β-carotene and lycopene. The resulting data underwent a principal component analysis (PCA).

Lycopene biosynthesis in *B. trispora* was investigated by quantitatively analyzing several compounds, including glycerol, alcohol, and phytoene (Fiehn et al., 2000; Roessner et al., 2001). An analysis of amino acid biosynthesis and carbon metabolism revealed that nicotine inhibited amino acid metabolism to almost undetectable levels, whereas many types of amino acids were detected during the fermentation without nicotine. Additionally, glucose consumption decreased following the addition of nicotine.

In the absence of nicotine, *B. trispora* accumulated 2.1 g/L β-carotene at 96 h, with 41.6 g/L dry cell

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weight (DCW) (Fig. 1a). Lycopene was biosynthesized after the addition of nicotine at 24 h, and 1.44 g/L lycopene was obtained upon completion of the fermentation, with 42.5 g/L DCW (Fig. 1b). The addition of 2.0 g/L nicotine completely inhibited β-carotene biosynthesis, but had no effect on the biomass (Fig. 1). Moreover, nicotine was consumed as the biomass increased. Thus, the nicotine level changed over time, essentially reaching 0 g/L at the end of the fermentation (i.e., completely consumed).

Regarding the fermentation without nicotine, a PCA revealed the first four principal components, amino acid metabolism, carbon metabolism, organic acid metabolism, and lipid metabolism, explained 46.2%, 12.5%, 9.7%, and 7.7% of the total variance, respectively. Additionally, a PCA of the lycopene fermentation process with nicotine indicated that the first four principal components, carbon metabolism, octadecenoic acid biosynthesis, organic acid metabolism, and hexadecenoic acid metabolism, explained 49.3%, 11.8%, 10.9%, and 8.5% of the total variance, respectively. Thus, the two fermentation processes differed significantly in terms of amino acid metabolism and lipid metabolism.

A comparison of the major metabolites revealed the main amino acids associated with the protein metabolism in the nicotine-free samples (Fig. 2a). Fatty acids were the most important components of the lycopene fermentation process with nicotine, which is consistent with the results of an earlier study (Tereshina et al., 2010). However, amino acid metabolism was almost completely inhibited.

The intracellular amino acids differed significantly between the two fermentation processes. In the absence of nicotine, the abundance of the main amino acids, including alanine, phenylalanine, α-aminoisobutyric acid, aspartic acid, glutamate, and glycine, exhibited a downward trend over the first 72 h of the fermentation, but then increased dramatically. This may have been because of the depletion of nitrogen as the biomass increased during the fermentation until 72 h, and when the biomass plateaued, the free amino acids accumulated. More importantly, glutamate was consumed after 84 h, implying that this amino acid is closely related to carotenoid biosynthesis. A previous study indicated that sodium glutamate promotes lycopene biosynthesis (Zhuang et al., 2007). However, during the lycopene fermentation with nicotine, few amino acids and their derivatives were detected, suggesting that nicotine decreases the efficiency of protein synthesis. Ding et al. (2014) also reported that nicotine decreases the amino acid concentration. This effect of nicotine may also contribute to fungal morphological changes. Specifically, because of a lack of a protein framework in the cell membrane, the *B. trispora* producing lycopene was relatively soft and fragile.

During the fermentation with nicotine, the intracellular glucose was underutilized (Fig. 2b), leading to the production of more glycerol than lycopene. Intracellular glycerol is reportedly an important compatible solute that adjusts cell membrane permeability by triggering the high osmolarity glycerol (HOG) pathway (Hohmann, 2002), which enables cells to adapt to hyperosmotic stress conditions (Dihazi et al., 2004; Petelenz-Kurdziel et al., 2013; Sabir et al., 2017).

The glycerol concentration during the fermentation with nicotine was relatively high during the whole process, but gradually decreased from 24 to

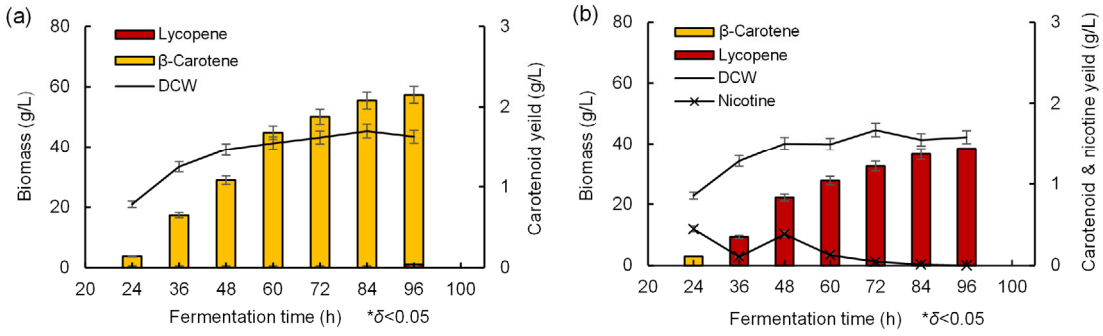

Fig. 1 Changes in fermentation processes due to nicotine

(a) Fermentation without nicotine; (b) Fermentation with nicotine. DCW: dry cell weight. Data are expressed as overall mean deviation δ (n=21)

72 h in the non-nicotine process (Fig. 2b). The most likely explanation for this observation is that the intracellular glycerol quickly increased to high levels via the HOG pathway to protect cells from withering due to the high sugar concentration, because glucose was nearly not consumed in the nicotine condition, and was kept in a relatively high concentration before 24 h in the fermentation without nicotine. The hyperosmotic stress declined along with the depletion of glucose as the fermentation progressed. The glycerol was also an intermediate of the metabolism of oil and was consumed in the middle fermentation stage. Thereafter, a metabolic collapse may have occurred during the apoptosis phase, during which the unconsumed glycerol accumulated gradually. Nicotine likely weakened the HOG pathway to some extent.

Fructose, which was not included as a media component, was converted to glucose, resulting in very low overall fructose levels during fermentation (Fig. 2b). Additionally, the fructose content exhibited the completely opposite trends in the two fermentation modes, implying that fructose metabolism is negatively correlated with the nicotine content.

Considerable changes were observed for some intermediates of the tricarboxylic acid (TCA) cycle (Fig. 2c), particularly fumarate, which exhibited the greatest differences between the two fermentation modes among the analyzed intermediates. The low fumarate level during the lycopene fermentation process with nicotine may indicate its importance for the nicotine-induced modifications of *B. trispora*. When nicotine was neutralized with organic acids, including fumarate and malate, the relevant metabolic flux and the TCA cycle flux decreased. Moreover, cellular damages due to nicotine were also expected to decrease.

Fumarate and malate concentrations remained relatively high from 36 to 60 h, but decreased at 72 h, which is approximately when the biomass peaked, before increasing sharply. During the main carotenoid biosynthesis stage, oxygen levels were apparently sufficient for cell respiration under the saccharified conditions. This may explain why the TCA cycle

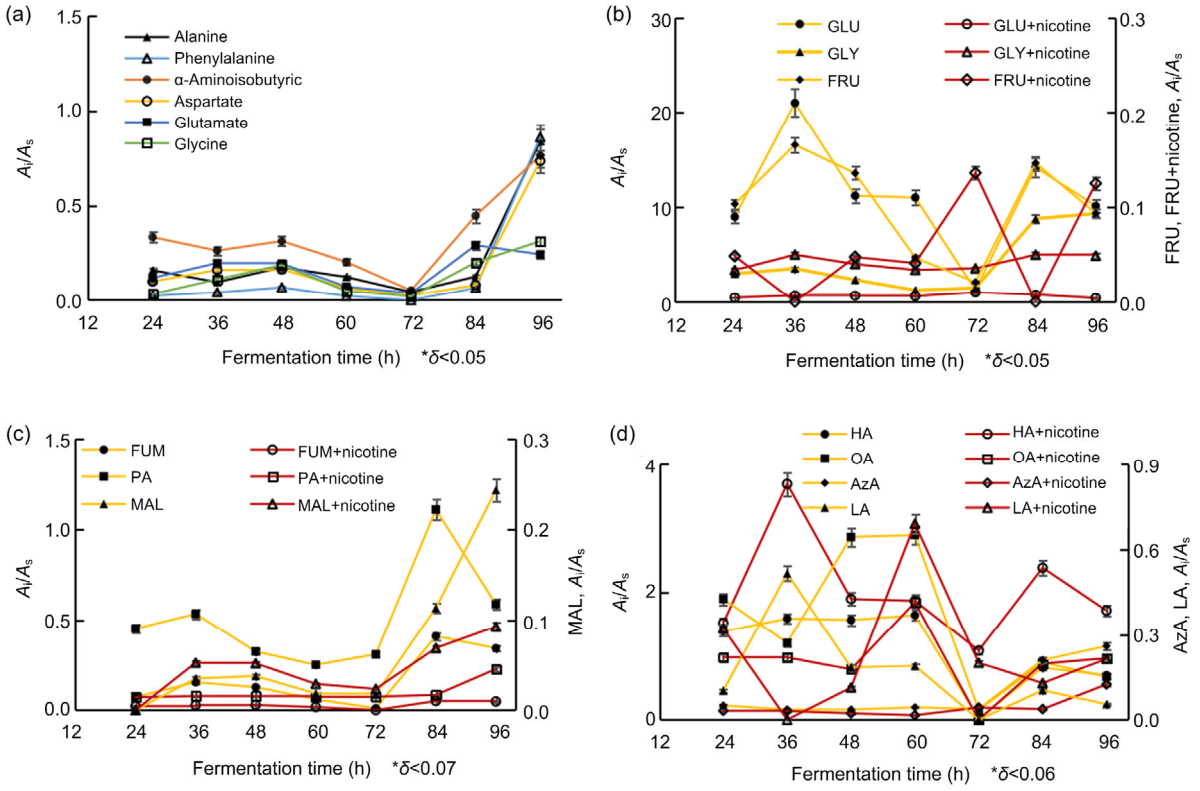

Fig. 2 Changes of metabolites in response to different fermentation modes

(a) Amino acids; (b) Carbonhydrate; (c) Organic acids; (e) Fatty acids. GLU: glucose; GLY: glycerol; FRU: fructose; FUM: fumarate; PA: phosphoric acid; MAL: malate; HA: hexadecenoic acid; OA: octadecenoic acid; AzA: azelaic acid; LA: linoleic acid; A_i/A_s: area of ingredient/area of standard. Data are expressed as overall mean deviation δ (n=21)

intermediates were highly abundant in this phase. Our findings are consistent with those of an earlier high-throughput metabolic state analysis (Villas-Bôas et al., 2005), in which the metabolic activity increased substantially after 72 h because of the lack of dissolved oxygen associated with an excessive biomass. Other studies proved that TCA cycle intermediates are maintained at high levels in anaerobic environments (Nissen et al., 1997; Franzén, 2003).

Phosphoric acid, which is usually important for adjusting signal transduction pathways, also contributes to the conversion of adenosine diphosphate (ADP) to adenosine triphosphate (ATP), the oxidation of citrate in the TCA cycle, and glycolysis (Ding et al., 2014). In the current study, an analysis of phosphoric acid (Fig. 2c) indicated that its intracellular content decreased considerably after the addition of nicotine at 24 h and remained low for the duration of the fermentation. When nicotine was not included in the fermentation process, phosphoric acid accumulated in part because of the highly active TCA cycle (Zhou et al., 2009). In the presence of nicotine, besides the normal metabolic response, phosphoric acid may have neutralized the nicotine, similar to fumarate.

Similar changes to the azelaic acid content were observed in both fermentation processes (Fig. 2d). Azelaic acid is considered to be a metabolic intermediate (Seo et al., 2018) that is a precursor for the biosynthesis of other fatty acids. The observed decrease in azelaic acid levels might be related to the carbon consumption during the fermentation phase. Increasing nicotine concentrations enhanced the accumulation of linoleic acid prior to the 60-h time-point, indicating that some glucose was used to biosynthesize linoleic acid instead of lycopene as well as hexadecenoic acid. Both of the unsaturated fatty acids were probably present in an intracellular oil depot to preserve lycopene and serve as terpene precursors.

The inclusion of nicotine during fermentation decreased the hexadecenoic acid and octadecenoic acid, but the hexadecenoic acid was kept relatively in a higher level (Fig. 2d). Recent studies confirmed that fatty acids promote the production of β-carotene and lycopene (Singh et al., 2018; Ma et al., 2019). In the current study, the fatty acid levels throughout the fermentation indicated that nicotine promoted the increase in the abundance of fatty acid derivatives. Additionally, the amino acid changes had almost the same tendencies as the changes to glycerol, glucose, malic acid, and sebacic acid, which may represent the flow of carbon sources. Glycerin can be used to synthesize hexadecenoic acid. During the fermentation process, β-carotene or lycopene should be efficiently synthesized until the 72-h time-point, after which they are degraded by the enzymes of the mevalonate pathway and other intermediates of carbon metabolism accumulate.

The GC-MS analysis described herein clarified the differences in the general *B. trispora* metabolite profiles between the fermentation of β-carotene without nicotine and the lycopene fermentation with nicotine as an inhibitor. The results of this study may form the basis for future investigations on the complex changes in the *B. trispora* metabolic network in response to nicotine. Some of the observed changes to metabolites are associated with the intracellular reactions mediating the response to nicotine during lycopene fermentation. Moreover, amino acid metabolism was mostly inhibited in the presence of nicotine. Glucose may be used during the normal β-carotene fermentation, but not in the lycopene fermentation with nicotine. The inclusion of nicotine maintained the TCA cycle at a relatively low level, with fatty acids and glycerin used as the main carbon sources. The metabolism of fatty acids, especially hexadecenoic acid and linoleic acid, apparently increases in response to nicotine. Future studies should focus on developing new *B. trispora* strains in which the metabolic network has been proficiently modulated to address industrial requirements (e.g. increased lycopene yield) based on proteomic and lipidomic research.

Contributors

Yang LIU and You-ran SHAO analyzed the data and wrote a draft of the manuscript. Zhi-ming WANG and Li-rong YANG conducted the fermentation experiments. Xiang-yu LI and Yu-zhou ZHANG completed the sample treatments and revised the description of the GC-MS analysis. Mian-bin WU and Jian-ming YAO designed the study and modified the manuscript. All authors have read and approved the final manuscript. Therefore, all authors have full access to all the data in the study and take responsibility for the integrity and security of the data.

Acknowledgments

We thank Liwen Bianji, Edanz Editing China (https://www.liwenbianji.cn/ac) for editing the English text of a draft of this manuscript.

Compliance with ethics guidelines

Yang LIU, You-ran SHAO, Xiang-yu LI, Zhi-ming WANG, Li-rong YANG, Yu-zhou ZHANG, Mian-bin WU, and Jian-ming YAO declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要

题　目：在尼古丁诱导下的三孢布拉霉产番茄红素代谢的分析

概　要：在三孢布拉霉发酵番茄红素过程中，阻断剂是必不可少的组分。尼古丁作为目前已知的最有效的番茄红素阻断剂之一，其在工业生产上具有巨大的实用价值。但阻断剂作为菌体的外源毒性生物碱，其对菌体的代谢势必有所改变。本文以尼古丁作为阻断剂，对其在三孢布拉霉发酵过程中的代谢影响做了初步研究。基于气相色谱-质谱（GC-MS）检测技术及小分子代谢组分的主成分分析（PCA），证明尼古丁在三孢布拉霉菌体内严重抑制氨基酸及糖代谢，同时脂肪酸和有机酸代谢也有所减弱，但相对强于糖代谢。因此，在尼古丁作为阻断剂时，为积累色素，碳源的补充应以脂肪酸或甘油为主。

关键词：代谢；番茄红素；尼古丁；三孢布拉霉；气相色谱-质谱