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Research paper

Intracellular amino and nonamino organic acids profiling of *Trichosporon cutaneum* on rich and limited nitrogen conditions for lipid production

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ABSTRACT

Trichosporon cutaneum is an oleaginous yeast capable of fermenting lignocellulosic sugars to produce lipid. Though numerous literature focused on lipid production under the condition of nitrogen source limitation, there is no information about intracellular metabolic changes involved in this process. In this study, metabolomic profiling of amino and nonamino organic acids was conducted to characterize the key metabolites and pathways. It was found that aspartic acid, ornithine, asparagine, glutamic acid, 4-amino-n-butyricacid, proline, lysine etc. were closely related to lipid production, and the pathway analysis showed that seven pathways may be related to lipid production with limited nitrogen source. This study provided a detailed dataset describing the changes of intracellular amino and nonamino organic acids when nitrogen was limited and lipid was accumulated, which added new knowledge on the mechanism of lipid accumulation and could be used for metabolic analysis of fermentation process as well as industrial strain improvement in the future.

1. Introduction

Trichosporon cutaneum is often found in industrial effluents or waste water containing phenols near refineries. It can accumulate a large amount of lipid and has strong ability to decompose phenolic compounds and adsorb heavy metals [1–3]. Recent years, with substantial attention turning towards lignocellulose, *T. cutaneum* is considered to be an ideal strain for production of microbial lipid from this cheap raw material because it exhibits strong resistance to the inhibitors such as formic acid, furan compounds, and aromatic compounds in the lignocellulosic hydrolysates [4,5]. Lipid consists of 80–90% triacylglycerols (TAG), and its synthesis requires continuous supplementation of fatty acids activated by coenzyme A (CoA) for glycerol back-bone acylation [6]. The synthesis of fatty acids in yeast is achieved by the constant supply of acetyl-CoA and formyl-CoA by fatty acid synthetase (FAS) in the cytoplasm. Acetyl-CoA produced by ATP citrate lyase (ACL) enters the cytoplasm via the malic acid-citric acid shuttle pathway. ACL consists of two subunits, ACL1 and ACL2, which are regulated by nitrogen sources in the environment. ACL is one of the important enzymes in the lipid synthesis. Formyl-CoA, synthesized by acetyl-CoA carboxylase (ACC), is the only source of carbon atoms synthesized from *de novo* lipid synthesis. The carbohydrates consumed by the cells are converted into the above precursors of lipid synthesis such as acetyl-CoA, formyl-CoA and glycerol, which are beneficial to the lipid synthesis [7,8]. However, carbohydrates are also metabolized to large amounts of other metabolites, e.g. amino acids and nonamino organic acids in the oleaginous yeasts [9]. These metabolites providing the raw materials for cell growth and maintaining the basic metabolism of cells are closely related to the formation of lipid precursors as well as the regulation of lipid synthesis. Up to date, there has been no publication focusing on cellular metabolic activity of *T. cutaneum* at metabolite level, even though there have been many reports on the metabolomics of lipid accumulation [10–13]. Metabolomics can be used to quantify a large number of intracellular metabolites at a particular time point and compare the changes of intracellular metabolites. Many analytical platforms are used for metabolomics, among which GC-MS is routinely employed because it is easier to use and has standardized reference library for identification of various peaks [14]. In addition, the methods of the leakage reduced cold glycerol-saline quenching and methyl chloroformate (MCF) derivatization have empowered the simultaneous and accurate analysis of numerous microbial metabolites. These sample pretreatment methods seem preferable for analyzing amino and nonamino organic acids in metabolomics studies [15]. Further optimization of the pretreatment methods based on the MCF derivatization has been completed in the previous studies [9].

Many studies show that oleaginous yeasts begin to accumulate lipid when certain elements in the medium are depleted but the excessive carbon source still exists [16–18]. The restriction of many elements can

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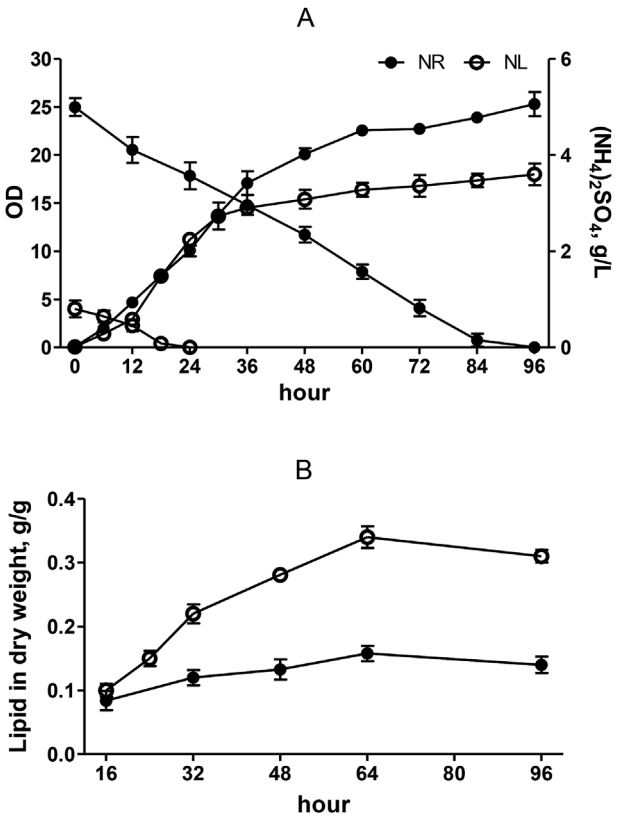

Fig. 1. Cultivation of *T. cutaneum* on rich nitrogen (NR) and limited nitrogen (NL) media. (A) Growth profiles, NH_4^+ (nitrogen) concentrations; (B) Lipid contents (g/g DCW).

promote the accumulation of lipid, in which nitrogen source limitation is the easiest to control and most commonly used [8]. In this study, the changes of intracellular amino and nonamino acids in *T. cutaneum* were studied at different nitrogen levels in the media. The study aimed at investigating the metabolic characteristics of these kinds of metabolites when lipid is produced by *T. cutaneum*. The results would lay foundations for future research on the efficient bioconversion of carbohydrates into lipid.

2. Materials and methods

2.1. Strains and culture conditions

The *T. cutaneum* strain ACCC20271 was purchased from China Agricultural Culture Collection Center. The strain was pre-cultivated in YPD medium containing (g/L) glucose 20, yeast extract 10 and peptone 20. The medium used for lipid production contained (g/L) glucose 30, KH_2PO_4 3, $MgSO_4$ 0.5, trace element solution 1% (v/v) and vitamin solution 0.1% (v/v). The composition of the trace element solution was as follows (g/L): EDTA 15, $MnCl_2{\cdot}4H_2O$ 1, $CuSO_4{\cdot}5H_2O$ 0.3, $CaCl_2{\cdot}2H_2O$ 4.5, $Na_2MoO_4{\cdot}2H_2O$ 0.4, H_3BO_3 1, KI 0.1, $CoCl_2{\cdot}6H_2O$ 0.3, $ZnSO_4{\cdot}7H_2O$ 4.5, $FeSO_4{\cdot}7H_2O$ 3. The composition of the vitamin solution was as follows (g/L): D-biotin 0.05, calcium pantothenate 1, nicotinic acid 1, thiamine hydrochloride 1, pyridoxine hydrochloride 1, para-aminobenzonic acid 0.2, myo-inositol 25. $(NH_4)_2SO_4$ was used as the nitrogen source, and its concentrations in rich nitrogen medium and limited nitrogen medium were 5 g/L and 0.8 g/L, respectively. Experiments were carried out in 250 mL conical flasks containing 50 mL medium inoculated with the pre-culture (initial OD_{600} = 0.05). The cultures were incubated in a rotary shaker at 220 rpm and 30 °C. Experiments were carried out in triplicate.

Table 1
Retention time, matching factors and reverse matching factors of identified metabolites using GC-MS.

Name	Retention time (min)	Match*	R.Match*
Hexanoic acid	7.443	929	929
Pyruvic acid	8.21	–	–
Fumaric acid	10.198	–	–
Malic acid	10.326	–	–
Succinic acid	10.625	–	–
Lactic acid	10.7	–	–
Octanoic acid	13.296	943	950
d_4-Alanine	13.546	–	–
Alanine	13.633	–	–
NADP/NADPH	14.582	939	945
2-aminobutyric acid	15.864	833	859
Valine	17.221	–	–
2-oxoglutaric acid	17.36	–	–
Phosphoenolpyruvic acid	18.824	871	871
Leucine	19.395	–	–
4-amino-n-butyric acid (GABA)	19.56	903	909
Isoleucine	19.727	–	–
Pyroglutamic acid	20.387	944	950
Proline	20.593	–	–
Asparagine	20.781	–	–
Cis-Aconitic acid	21.887	–	–
Aspartic acid	22.557	–	–
Citric acid	22.669	–	–
S-Adenosyl-L-Homocysteine	23.607	921	903
Serine	24.202	–	–
Glutamic acid	25.565	–	–
Methionine	25.748	–	–
Glyceric acid	26.244	709	752
D-2-amino adipic acid	27.856	873	873
Phenylalanine	28.3	–	–
Myristic acid	28.465	923	923
Isocitric acid	28.594	859	859
Palmitoleic acid	32.085	945	945
Ornithine	32.243	892	901
Palmitic acid	32.316	941	941
Lysine	33.458	–	–
Histidine	34.273	–	–
Linoleic acid	34.402	920	940
Oleic acid	34.689	957	957
Stearic acid	34.85	927	927
Tyrosine	35.643	–	–
Cystathionine	39.335	858	858

*: for a perfect match, this value is 1000. The matching factors and reverse matching factors of the metabolites identified by NIST Mass Spectra Search Program.
-: identified by authentic compounds.

2.2. Sample preparation for metabolomics analysis

The cells in 7 mL culture aliquots were quenched with 28 mL cold glycerol-saline solution according to our previous study [9]. The quenching solution was prepared with 13.5 g/L sodium chloride solution and glycerol to a final ratio of 2:3 (v/v) and precooled at −20 °C overnight. The cells were separated by centrifugation at −15 °C and washed once using the quenching solution. Internal standard (0.2 μmol of d_4-alanine) was added to each sample before extraction. Then, the intracellular metabolites were extracted using cold methanol solution (50%, v/v), and the mixture was undergone three freeze-thaw cycles [19]. Finally, the solvent was removed by the evaporation system Speed Vac SPD131DDA (Thermo Scientific, Waltham, MA). Samples were prepared in quadruplicate.

2.3. Sample derivatization for GC-MS analysis

The freeze-dried samples were derivatized using the methyl chloroformate (MCF) method which was achieved according to the method of Villas-Bôas et al. [20].