Metabolic Engineering of *Issatchenkia orientalis* for succinic acid production

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The nonconventional yeast *Issatchenkia orientalis* is an industrially relevant platform microorganism for production of organic acids thanks to its unusual ability to grow in highly acidic conditions and tolerance to high concentrations of several organic acids. Previously, introduction of a reductive TCA pathway into the yeast enabled succinic acid production at titer of 11.6 g/L in shake flask fermentations. Here we describe further metabolic engineering efforts to enhance the production of succinic acid in *I. orientalis*. Further genetic optimizations, including deletion of by-product pathways and heterologous expression of a dicarboxylic acid transporter, allowed 24.1 g/L of succinic acid to be produced from 50 g/L of glucose in shake flask fermentation. Nevertheless, pyruvic acid accumulated at titer of 19.8 g/L because of the lack of cytosolic NADH, which hindered further production of succinic acid. To increase cytosolic NADH availability, glycerol was used as co-substrate. Fermentation using 50 g/L of glucose and 20 g/L of glycerol allowed conversion of the accumulated pyruvic acid to succinic acid and attained a titer of 38.6 g/L of succinic acid. Deletion of a dicarboxylic acid importer and overexpression of the glycerol consumption pathway further improved the titer of succinic acid to 42.1 g/L. In fed-batch fermentation in which pH was set at 3, the best strain could produce succinic acid at titer of 103 g/L, yield of 0.56 g/g glucose equivalent, and productivity of 0.43 g/L/h from pure glucose and glycerol and at titer of 120 g/L and productivity of 0.56 g/L/h from sugarcane juice and glycerol. This is the highest titer achieved in minimal media at low pH 3 to date from a recombinant organism. Overall, this work shows that *I. orientalis* can serve as a potential industrial platform for production of succinic acid.