Bioresource Technology 102 (2011) 6982–6989

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Simultaneous production of citric acid and invertase by *Yarrowia lipolytica SUC*$^+$ transformants

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ARTICLE INFO

Article history:
Received 4 February 2011
Received in revised form 28 March 2011
Accepted 9 April 2011
Available online 15 April 2011

Keywords:
Citric acid
Invertase
Yarrowia lipolytica
Sucrose
Glycerol

ABSTRACT

Simultaneous production of citric acid (CA) and invertase by *Yarrowia lipolytica* A-101-B56-5 (*SUC*$^+$ clone) growing from sucrose, mixture of glucose and fructose, glucose or glycerol was investigated. Among the tested substrates the highest concentration of CA was reached from glycerol (57.15 g/L) with high yield ($Y_{CA/S}$ = 0.6 g/g). When sucrose was used, comparable amount of CA was secreted (45 g/L) with slightly higher yield ($Y_{CA/S}$ = 0.643 g/g). In all cultures amount of isocitrate (ICA) was below 2% of total citrates. Considering invertase production, the best carbon source appeared to be sucrose (72 380 U/L). The highest yield of CA and invertase biosynthesis calculated for 1 g of biomass was obtained for cells growing from glycerol (9.9 g/g and 4325 U/g, respectively). Concentrates of extra- and intracellular invertase of the highest activity were obtained from sucrose as substrate (0.5 and 1.8 × 10^6 U/L, respectively).

1. Introduction

The world's annual demand of citric acid (CA) exceeds 1.4 million tons (Papanikolaou et al., 2009). This acid is commercially used as an acidity regulator and flavor enhancer in food industry, particularly in the production of beverages, as well as in pharmaceutical manufacturing (Vandenberghe et al., 2000). The most commonly used microorganism for industrial production of CA is filamentous fungus *Aspergillus niger*, which is grown in solid-state or submerged fermentation systems (Pazouki et al., 2000; Shojaosadati and Babaeipour, 2002; Vandenberghe et al., 2000). This process can be performed using cane and beet molasses, starch and unusual carbon sources such as coffee husk or cassava bagasse. Recently an alternative technology for the production of CA based on *Yarrowia lipolytica* yeast has been developed (Kamzolova et al., 2003; Papanikolaou et al., 2008; Robak et al., 2007; Rymowicz et al., 2010). Apart from CA, in cultures performed under nitrogen limitation (required for CA secretion), some strains of *Y. lipolytica* are able to produce intracellular lipids (André et al., 2009; Papanikolaou et al., 2009; Makri et al., 2010).

Naturally occurring strains of these yeasts are able to grow in media containing glucose, fructose, ethanol, glycerol and some raw materials: glucose hydrol, vegetable oils, fatty acids and raw glycerol (Arzumanov et al., 2000; Darvishi et al., 2009; Papanikolaou et al., 2008). Because of its very low price, glycerol became of particular interest to researchers involved in the production of CA by the yeast *Y. lipolytica*. Crude glycerol is formed as the main waste product during the process of biodiesel production. Apart from glycerol, the waste product includes oil residues, free fatty acids (about 1%) and salts (sodium and potassium) which make it very good substrate for the production of CA (André et al., 2009; Rymowicz et al., 2010). Inability of *Y. lipolytica* wild strains to grow in media containing sucrose as carbon source precludes the possibility of using another cheap waste material which is molasses. For economical aspects it became important to improve *Y. lipolytica* strains by genetic engineering. Strains containing the *SUC2* gene from the yeast *Saccharomyces cerevisiae*, which encodes the enzyme invertase (EC 3.2.1.26), were generated by Nicaud et al. (1989), Förster et al. (2007), and Walczak and Robak (2009). These strains are able to grow and secrete CA from pure sucrose or sugar found in significant amounts in beet molasses. Also combined cultures, in which one of the strains possessed the invertase gene, allowed the use of sucrose as substrate for CA biosynthesis (Żarowska et al., 2001).

Abbreviations: CA, citric acid; ICA, isocitric acid; EI, extracellular invertase activity; II, intracellular invertase activity; Q_{CA}, citric acid volumetric productivity; q_{CA}, specific rate of citric acid production; Q_{EI}, extracellular invertase volumetric productivity; q_{EI}, specific rate of extracellular invertase production; Q_{II}, intracellular invertase volumetric productivity; q_{II}, specific rate of intracellular invertase production; X, biomass; $Y_{CA/S}$, citric acid yield from substrate; $Y_{CA/X}$, citric acid yield from biomass; $Y_{EI/S}$, extracellular invertase yield from substrate; $Y_{EI/X}$, extracellular invertase yield from biomass; $Y_{II/S}$, intracellular invertase yield from substrate; $Y_{II/X}$, intracellular invertase yield from biomass.

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doi:10.1016/j.biortech.2011.04.032

Invertase (β-fructofuranosidase) is present in bacteria, yeasts, fungi, higher plants and in some animal cells (Rubio et al., 2002; Rubio and Navarro, 2006; Ul-Haq et al., 2008; Kotwal and Shankar, 2009). Commercially this enzyme is extracted from baker's yeast biomass (Hustedt et al., 1990). Invertase is being widely applied in food industry, particularly in the production of the chocolates with liquid center. Annually, only in one region of Poland (Silesia) around 900 kg of the invertase preparation are used (personal communication). At high concentration of sucrose this enzyme shows transglycosidase activity and thus can be used in the production of the fructooligosaccharides (FOS) – sugars with prebiotic properties (Ning et al., 2010). It is worth to mention, that invertase is also used in the production of artificial honey, plasticizing agents used in cosmetics, medicines and paper industry. Recently, it has found the application in the construction of electrodes for detection of sucrose level (Kotwal and Shankar, 2009).

Due to the ability of *Y. lipolytica SUC*$^+$ transformants for CA secretion and invertase production, it will be interesting to establish an environmentally friendly process leading to simultaneous synthesis of both metabolites, especially in media containing easily available and cheap carbon sources. For this purpose, we conducted bioreactor batch cultures (BC) of some *Y. lipolytica* A-101 *SUC*$^+$ transformants as processes of those two products biosynthesis from glycerol or sucrose in comparison to the same processes with glucose or glucose and fructose as control substrates.

2. Methods

2.1. Microorganisms

Y. lipolytica A-101 wild type strain (WT) and its *SUC*$^+$ transformants (*Y. lipolytica* A-101: A18, B1-1, B9-2, B54-6, B55-3, B56-5, B57-4, B60-4, K10) used in this study, came from the collection belonging to the Department of Biotechnology and Food Microbiology, Wroclaw University of Environmental and Life Sciences, Poland. *Y. lipolytica SUC*$^+$ transformants were obtained by Walczak and Robak (2009). Yeast strains were stored on YM agar plate at 4 °C.

2.2. Media and culture conditions

2.2.1. Bioscreen C

Microcultures (300 μL) of *Y. lipolytica* strains were performed using Bioscreen C (Oy Growth Curves Ab Ltd., Finland) in MMTS medium containing in 1 L of distilled water: sucrose 10.0 g, NH_4Cl 5.0 g, KH_2PO_4 2.5 g, $MgSO_4 \times 7H_2O$ 1.0 g, thiamine 3×10^{-6} g, uracil 0.02 g (when necessary), mineral salts stock solution 1 mL. Mineral salts stock solution contained in 1 L: $Ca(NO_3)_2 \times 4H_2O$ 20.0 g, $FeCl_3 \times 6H_2O$ 2.0 g, H_3BO_3 0.5 g, $MnSO_4 \times 4H_2O$ 0.4 g, $ZnSO_4 \times 7H_2O$ 0.4 g, Na_2MoO_4 0.2 g, $CuSO_4 \times 5H_2O$ 0.1 g, $CoCl_2$ 0.1 g, KI 0.1 g. For induction of *XPR2* promoter 0.1 or 0.01% of peptone was added or medium was prepared on 50 mM phosphate buffer, pH 6.0. The inoculum consisted of 50 μL of 24 h shaken yeast culture in YPG medium standardized to 4×10^6 cells/mL. Microcultures were carried out at 25 °C during 72–96 h with constant shaking and measuring of optical density (420–580 nm) every 15 min.

2.2.2. Cultures for nucleic acids extraction

For extraction of nucleic acids (DNA and RNA), yeast strains *Y. lipolytica* A-101: WT, A18, B54-6, B56-5 and B57-4 were grown in 100 mL flasks containing 20 mL of MMTG medium similar to MMTS, where instead of sucrose, 10 g/L of glucose was added. The cultures were carried out for 24 h in HT incubator shaker (INFORS-HT, Bottmingen, Switzerland) at 28 °C with shaking rate 170 rpm and amplitude 7 mm.

For genomic DNA extraction 1 mL of culture was transferred to 19 mL of fresh MMT (medium without carbon source) and incubated for 24 h, as described previously. Subsequently, frame flasks were transferred to 4 °C and again incubated for 24 h. In these starving conditions the majority of the cells have been synchronized and kept in the G0/G1 phase of the cell cycle. After incubation, cultures were centrifuged (Universal 32R, Hettich, Tuttlingen, Germany) at 4 °C for 5 min, 15 000 rpm, washed twice with 20 mL of sterile distilled water and suspended in 2 mL of water. Amount of yeast cells was standardized and for DNA extraction 9.5×10^7 of cells has been taken.

For RNA extraction, the MMTG culture was centrifuged for 5 min, 15 000 rpm at room temperature (Universal 32R, Hettich, Tuttlingen, Germany). Supernatant was discarded and biomass was washed twice with 20 mL of sterile distilled water and suspended in 20 mL of MMTS medium (50 g/L of sucrose as carbon source) and incubated for 3 h. After incubation OD_{600} was measured using BioPhotometer Plus (Eppendorf, Hamburg, Germany) and standardized to 1.5 with sterile distilled water.

2.2.3. Bioreactor study

CA and invertase biosynthesis, as batch cultures (BC) were carried out during 72 h in 5 L stirred-tank reactors BIO-STAT B-PLUS (Sartorius, Frankfurt, Germany) with working volume 2 L at 30 °C, 800 rpm and aeration rate 0.36 vvm. Production media contained in 1 L of tap water: sucrose 100 g, NH_4Cl 1.5 g, KH_2PO_4 0.7 g, $MgSO_4 \times 7H_2O$ 1.0 g, YE 0.3 g, thiamine 3×10^{-6} g, uracil 0.02 g (when necessary). Culture acidity was automatically controlled at pH 6.8 using 40% (w/v) NaOH solution. Inocula consisted of 10% of total working volume. The inoculum medium contained in 1 L of tap water: sucrose 50 g, NH_4Cl 1.5 g, YE 1.0 g, peptone 1.0 g, uracil 0.02 g (when necessary). The cultures were grown in 0.5 L flasks containing 0.1 L of medium on a rotary shaker (Elpan, Poznań, Poland), 170 rpm, 28 °C, 48 h.

In the second step of bioreactor studies sucrose, glycerol, glucose or glucose with fructose (1:1) were used as carbon sources. Inocula and production media were prepared with the same substrate. Culture conditions were as described above.

Glucose, fructose and sucrose used in this study were commercially available in the grocer's shop, whereas glycerol was of technical grade. Sucrose, used for strains screening (Table 1) and substrates screening (Table 2), came from two different producers.

Osmolality of media was measured using Marcel *OS3000* osmometer (Marcel Ltd., Zielonka, Poland) and reached in mOsmol/kg the value of: 680 for glucose, 686 for glucose + fructose, 410 for sucrose and 1351 for glycerol based medium.

For analysis 15 mL samples were taken and centrifuged 10 min at 5000 rpm using 3–16 K centrifuge (Sigma, St. Louis, MO, USA). Supernatant and cells sediment were collected and used for further analysis. The first samples (time 0) were taken 10 min after culture inoculation and the time of the next samples collection is indicated on the figures.

2.3. Analytical methods

2.3.1. Biomass determination, ultrafiltration and intracellular invertase extraction

For dry biomass determination the cells sediment from 15 mL culture sample was washed twice with distilled water, filtered on 0.45 μm pore-size membrane and dried at 105 °C to the constant weight using weight-dryer WPS 110S (Radwag, Poznań, Poland).

For kinetic analysis of intracellular invertase, enzyme was extracted by sonication of collected cells samples (15 min, amplitude 100%, 0.5 s intervals) using SONOPLUS HD 2070 ultrasonic

Table 1
Biomass, CA, ICA, extra- and intra-cellular invertase production parameters in BC of *SUC*$^+$ clones of *Y. lipolytica* A-101.

Clone	X (g/L)	CA (g/L)	$Y_{CA/S}$ (g/g)	$Y_{CA/X}$ (g/g)	Q_{CA} (g/L/h)	q_{CA} (g/g/h)	ICA		EI (U/L)	$Y_{EI/S}$ (U/g)	$Y_{EI/X}$ (U/g)	Q_{EI} (U/L/h)	q_{EI} (U/g/h)	II[a] (U/L)	$Y_{II/S}$ (U/g)	$Y_{II/X}$ (U/g)	Q_{II} (U/L/h)	q_{II} (U/g/h)
							(g/L)	(%)										
Homologous transformants																		
A18	8.4 ± 0.30	44.76 ± 0.90	0.57 ± 0.04	5.33 ± 0.23	0.602 ± 0.035	0.072 ± 0.003	8.06 ± 0.48	15.26	664 ± 84	8.58 ± 1.09	79.03 ± 9.48	9.09 ± 0.89	1.08 ± 0.12	3392 ± 305	115.4 ± 14.65	403.8 ± 36.34	46.46 ± 5.57	5.53 ± 0.49
B54-6	5.2 ± 0.23	5.36 ± 0.11	0.17 ± 0.01	1.03 ± 0.23	0.071 ± 0.003	0.014 ± 0.002	0.88 ± 0.05	14.10	451 ± 33	14.74 ± 0.06	86.68 ± 6.29	6.74 ± 0.47	1.3 ± 0.09	663 ± 50	54.21 ± 3.52	127.5 ± 9.56	9.918 ± 0.64	1.91 ± 0.13
B55-3	3.2 ± 0.19	0.55 ± 0.05	0.03 ± 0.00	0.17 ± 0.01	0.007 ± 0.000	0.002 ± 0.000	0 ± 0.00	0	170 ± 11	8.08 ± 0.53	53.08 ± 3.31	2.32 ± 0.19	0.73 ± 0.04	67 ± 4	8.083 ± 0.26	20.83 ± 1.31	0.926 ± 0.03	0.29 ± 0.02
B57-4	6 ± 0.39	31.71 ± 0.63	0.56 ± 0.05	5.29 ± 0.91	0.434 ± 0.042	0.072 ± 0.011	0.79 ± 0.05	2.43	556 ± 27	9.25 ± 0.46	92.59 ± 4.51	7.61 ± 0.64	1.27 ± 0.06	2237 ± 107	92.89 ± 4.64	372.8 ± 17.89	30.64 ± 1.53	5.11 ± 0.25
Heterologous transformants																		
B56-5	9.7 ± 0.31	46.2 ± 0.92	0.55 ± 0.03	4.76 ± 0.25	0.626 ± 0.028	0.065 ± 0.009	0.95 ± 0.06	2.01	3720 ± 212	41.08 ± 2.60	383.3 ± 21.76	47.36 ± 3.55	4.88 ± 0.30	25 404 ± 1245	550.6 ± 34.68	2619 ± 129.5	345.4 ± 21.76	35.61 ± 1.78
B60-4	6.4 ± 0.11	22.65 ± 0.45	0.27 ± 0.02	3.54 ± 0.23	0.314 ± 0.022	0.049 ± 0.004	0.99 ± 0.06	4.19	2111 ± 122	25.16 ± 1.43	329.9 ± 19.06	29.32 ± 2.19	4.58 ± 0.26	890 ± 52	26.53 ± 1.51	139.1 ± 8.06	12.37 ± 0.71	1.93 ± 0.11
K10	5.2 ± 0.23	33.3 ± 0.67	0.44 ± 0.04	6.4 ± 0.48	0.456 ± 0.027	0.088 ± 0.005	0.94 ± 0.05	2.75	1356 ± 61	18.04 ± 2.08	260.7 ± 11.62	18.83 ± 1.41	3.62 ± 0.16	2155 ± 95	71.73 ± 7.25	414.5 ± 18.44	29.94 ± 3.02	5.76 ± 0.26

[a] Obtained by biomass sonication and centrifugation.

homogenizer (Bandelin GmbH & Co. KG, Berlin, Germany) followed by centrifugation (conditions as described above).

For invertase extraction the collected by centrifugation biomass (from whole bioreactor cultures) was washed twice with distilled water, suspended in 500 mL of 0.1 M $NaHCO_3$ (baking soda) followed by 24 h incubation at 40 °C and 24 h at 4 °C and finally centrifuged for 30 min, at 5 000 rpm, 4 °C. Obtained supernatant (cell's extract) was concentrated by ultrafiltration and contained intracellular invertase. Cells debris could be utilized as animals feed supplement because they did not contain any toxin.

The post culture media and cell's extracts were concentrated using Labscale™ TFF Ultrafiltration System (Millipore, Billerica, MA, USA) with Pellicon XL Biomax 50 cassette. The whole procedure was carried out at 4 °C, with inlet pressure of 3 bar and outlet pressure of 1 bar to the final volume of approximately 50 mL (limited by samples viscosity).

2.3.2. Sugars, glycerol and acids measurement

CA, glucose, fructose, sucrose and glycerol were determined by HPLC (Beckman Gold System, Brea, USA) using Aminex HPX87H column coupled to a UV (210 nm) and RI detectors. The column was eluted with 0.01 N H_2SO_4 at room temperature and flow rate of 0.6 mL/min. Sugars, glycerol and CA were identified and quantified with reference to standards. Before the HPLC analysis samples were filtered on 0.45 μm pore-size membranes.

Isocitric acid was analyzed using enzymatic method described by Goldberg and Ellis (1983).

2.3.3. Invertase activity and protein determination

Extra- and intracellular invertase activity was measured in post-culture media and cell's extracts. The reaction mixture in final volume of 0.5 mL contained 0.1 M sucrose in 0.1 M acetate buffer (pH 5.0) and appropriately diluted post-culture media or cell's extracts and was incubated for 10 min at 37 °C. Enzyme catalyzed reaction was stopped by the addition of 1.5 mL of 3,5-dinitrosalicylic acid (DNS) and the released reducing sugars were determined according to Miller (1959). One unit of activity (U) was defined as the amount of enzyme releasing 1 μmol of reducing sugars per minute in assay's conditions. Specific activity was calculated per mg of protein. Protein concentration was determined according to Lowry method (Lowry et al., 1951) with bovine serum albumin (BSA) as standard.

Before the measurement of invertase activity in post-culture media, samples were dialyzed against 0.1 M acetate buffer (pH 5.0) during 24 h at 4 °C (to remove residual sugars).

2.3.4. Reverse transcription and qRT-PCR

Genomic DNA was prepared according to Hoffman and Winston (1987) and RNA extraction was performed with TRIzol®Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The amount of nucleic acids was measured on ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). The cDNA was prepared using Reverse Transcription System (Promega, Fitchburg, WI, USA) according to the manufacturer's instruction and purified with the QIAquick™ Nucleotide Removal Kit (QIAGEN, Venlo, Netherlands).

Real Time PCR was performed using the Light Cycler® FastStart DNA Master SYBR Green I (Roche, Penzberg, Germany) with 0.5 μM forward and reverse primer and 1 μg of the template (cDNA or genomic DNA) in a final reaction volume of 10 μL. Thermocycling was performed in a Light Cycler® 2.0 (Roche, Penzberg, Germany) initiated by a 10 min incubation at 95 °C, followed by 40 cycles of 10 s at 95 °C, 5 s at 61 °C, 10 s at 72 °C for *SUC2* (invertase gene) and 10 s at 95 °C, 5 s at 62 °C, 8 s at 72 °C for *ACT1* (actin, reference gene). Fluorescence data were acquired

Table 2
Biomass, CA, ICA, extra- and intracellular invertase production parameters in BC of *Y. lipolytica* A-101-B56-5 from different substrates.

Substrate	X (g/L)	CA (g/L)	$Y_{CA/S}$ (g/g)	$Y_{CA/X}$ (g/g)	Q_{CA} (g/L/h)	q_{CA} (g/g/h)	ICA (g/L)	ICA (%)	EI (U/L)	$Y_{EI/S}$ (U/g)	$Y_{EI/X}$ (U/g)	Q_{EI} (U/L/h)	q_{EI} (U/g/h)	II[a] (U/L)	$Y_{II/S}$ (U/g)	$Y_{II/X}$ (U/g)	Q_{II} (U/L/h)	q_{II} (U/g/h)
Glucose	7.8 ± 0.26	46.25 ± 1.16	0.499 ± 0.031	5.929 ± 0.19	0.639 ± 0.016	0.082 ± 0.003	0.45 ± 0.03	0.96	2599 ± 93	27.83 ± 1.17	333.2 ± 11.72	35.66 ± 2.84	4.57 ± 0.41	26 857 ± 691	287.5 ± 19.78	3443 ± 80	368.4 ± 23.50	47.23 ± 1.63
Glucose + Fructose	9.8 ± 0.21	49.60 ± 1.24	0.564 ± 0.025	5.060 ± 0.09	0.689 ± 0.017	0.070 ± 0.001	0.41 ± 0.02	0.82	2273 ± 102	25.24 ± 1.06	231.9 ± 10.15	30.81 ± 2.15	3.14 ± 0.13	27 920 ± 2177	317.9 ± 41.40	2849 ± 221	388.0 ± 23.97	39.59 ± 4.06
Sucrose	12.4 ± 0.43	45.02 ± 1.12	0.643 ± 0.033	3.630 ± 0.13	0.625 ± 0.016	0.050 ± 0.002	0.39 ± 0.02	0.86	3253 ± 88	46.00 ± 2.67	262.3 ± 7.12	44.70 ± 2.54	3.60 ± 0.51	31 841 ± 2101	454.7 ± 37.74	2568 ± 169	441.8 ± 45.08	35.63 ± 4.43
Glycerol	5.8 ± 0.31	57.15 ± 1.43	0.600 ± 0.027	9.853 ± 0.57	0.794 ± 0.020	0.137 ± 0.008	0.40 ± 0.02	0.70	3416 ± 232	35.77 ± 1.25	589.0 ± 40.09	47.34 ± 2.50	8.16 ± 0.62	21 666 ± 1234	227.1 ± 23.68	3736 ± 211	3006 ± 9.31	51.83 ± 5.03

[a] Obtained by biomass sonication and centrifugation.

during each elongation step. Specificity was controlled by melting curves analysis performed at the end of each run. Invertase expression was detected with the following primers: SUC2-fwd 5′-TGCCTTTGTCCCAACTAACC-3′ and SUC2-rev 5′-GAGACCAGGGACCAGCATTA-3′. Expression of actin reference gene was detected with the specific primers: ACT1-fwd 5′-TCCAGGCCGTCCTCTCCC-3′ and ACT1-rev 5′-GGCCAGCCATATCGAGTCG-3′. The results were analyzed according to ddC_T method (Schmittgen and Livak, 2008).

All the experiments included in this study were performed in triplicates.

3. Results and discussion

Preliminary screening of the best CA and invertase producer was based on the ability of transformants to grow in MMT medium with sucrose as sole carbon source (supplemented with uracil when necessary) checked in Bioscreen C (data not shown). In general, better growth was observed for transformants with heterologous integration of SUC2 expression cassette (*SUC*$^+$ *URA*$^+$). Two out of six tested clones, presenting homologous integration of the cassette (*SUC*$^+$ *URA*$^-$), *Y. lipolytica* A-101-B1-1 and A-101-B9-2, grew poorly and were not included for further analysis.

Seven transformants were tested for CA and invertase production in bioreactor studies under nitrogen limitation in medium containing sucrose as carbon source (Table 1). The highest amount of CA (44.8–46.2 g/L), with a high yield (0.55–0.57 g/g of sucrose), was reached in BC for two strains: *Y. lipolytica* A-101-A18 and *Y. lipolytica* A-101-B56-5. BC process based on the same strains presented also the highest productivity of this metabolite (0.602–0.626 g/L/h). However, the clone *Y. lipolytica* A-101-A18 accumulated up to 15% of isocitric acid, which was an undesired metabolite. It needs to be underlined that transformant *Y. lipolytica* A-101-B56-5 accumulated very little amount of ICA, which accounted for 0.7–2% of total citric acids, regardless of used substrate. So far, higher amounts of this undesired metabolite have been reported in the literature (Moeller et al., 2007, 2010; Darvishi et al., 2009). Also the wild strain *Y. lipolytica* A-101, some of its acetate mutants and *SUC*$^+$ transformants accumulated 4–21% of ICA, (Wojtatowicz et al., 1997; Förster et al., 2007; Rywińska et al., 2010). In some cases, under nitrogen limitation, some of *Y. lipolytica* strains are able to accumulate high amount of intracellular lipids (André et al., 2009; Papanikolaou et al., 2009; Makri et al., 2010). CA and intracellular lipids production are closely connected. When the concentration of CA in the cytosol increases, it could be either converted to cellular fatty acids or secreted into the culture medium.

The next important parameter of selection for simultaneous process establishment was the yield of invertase production. In this case, the highest values were reached with *Y. lipolytica* A-101-B56-5 strain. It was not surprising because, as we have shown by Real Time PCR, two copies of SUC2 gene were integrated into its genome (Fig. 1). The analysis carried out on cDNA as well as on genomic DNA did not show any differences in the expression of this gene on transcriptional level. The SUC2 gene was cloned into the yeast genome under the control of XPR2 promoter, whose expression requires the presence of peptone in the medium and pH 6.0. Nicaud et al. (1989) and Förster et al. (2007) suggested, that for induction of the promoter a dose of peptone from 0.17% to 0.5% is required, while Walczak and Robak (2009) have shown that the growth of *SUC*$^+$ transformants of *Y. lipolytica* A-101 in the medium with sucrose occurs already in the presence of 0.1% and even of 0.01% of peptone. For this reason, in the current study yeast extract (component of the

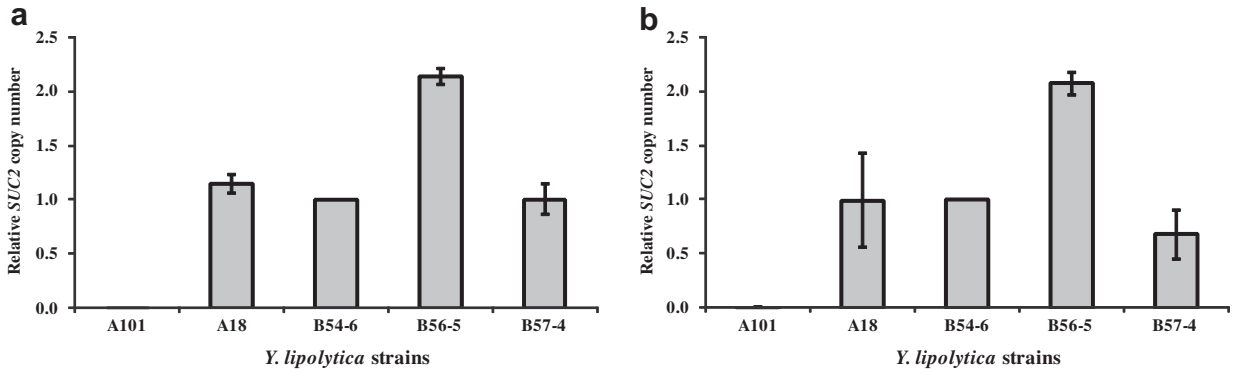

Fig. 1. Expression of *SUC2* gene in *Y. lipolytica* A-101 transformants normalized to actin gene with (a) genomic DNA, (b) cDNA as template.

medium, see Section 2) was a sufficient source of promoter inductor (about 0.02% of peptone).

In further BC studies transformant *Y. lipolytica* A-101-B56-5 appeared to be a good producer of CA from all used substrates (Table 2). Final content of this metabolite in the medium was between 45.02 and 57.15 g/L with a yield from 0.499 to 0.643 g/g of the substrate. The highest yield of CA was obtained using sucrose while the highest amount using glycerol as substrate. As reported for other *Y. lipolytica* strains, the use of glycerol in feed batch culture allowed to obtain slightly lower concentration of CA (33.5 g/L) with comparable yield (0.52 g/g) from that substrate (Makri et al., 2010). Moeller et al. (2007) obtained comparable amount of CA (41 g/L) with similar yield (0.55 g/g) in BC process with glucose. Acetate negative strains of *Y. lipolytica* A-101 during BC with high glycerol concentration accumulated higher level of this metabolite (66–80 g/L) with comparable yield (0.40–0.53 g/g) (Rywińska et al., 2010). The CA biosynthesis process performed by those mutants in cell recycling system or feed-batch culture and high concentrations of glycerol in the culture medium resulted in the increased amount of CA (107–124 g/L) and yield amounting to 0.64–0.77 g/g (Rymowicz et al., 2010). Higher amount of CA (140 g/L) with high yield from substrate (0.82 g/g) was obtained from sucrose by the *SUC*$^+$ strain *Y. lipolytica* H222-S4 T5 during feed batch culture (Förster et al., 2007). Comparable yield of this acid (0.85 g/g) was obtained from glucose (Papanikolaou et al., 2009) and by supplementation of glucose based medium with sodium acetate (Robak et al., 2007). Higher yield of CA was reached when ethanol was used as substrate (Arzumanov et al., 2000; Kamzolova et al., 2003).

CA biosynthesis productivity obtained using *Y. lipolytica* A-101-B56-5 ranged from 0.63 to 0.79 g/L/h (Table 2). The highest value was reached for the process using glycerol as substrate. Similar values of CA productivity from glucose, sucrose and glycerol are confirmed in the literature (Förster et al., 2007; Moeller et al., 2010; Rywińska et al., 2010). Whereas, increased productivity of this metabolite (0.85–1.42 g/L/h) from crude glycerol was observed in feed-batch fermentation and cell recycling process (Rymowicz et al., 2010). Even higher results (1.70 g/L/h) were obtained from glucose in repeated batch culture (Moeller et al., 2010).

The highest value of CA specific production rate for the *Y. lipolytica* A-101-B56-5 clone, was obtained in medium with glycerol, while the lowest one was acquired in the process with sucrose: 0.14 and 0.05 g/g/h, respectively (Table 2). Comparable results of CA specific production rate were presented for *Y. lipolytica* VKM Y-2373 (0.138 g/g/h) and *Y. lipolytica* N1 (0.120 g/g/h) growing from ethanol (Arzumanov et al., 2000; Kamzolova et al., 2003) as well as for *SUC*$^+$ *Y. lipolytica* H222-S4 T5 growing from sucrose (0.091 g/g/h) (Förster et al., 2007). More than three times lower values of this parameter (0.03–0.043 g/g/h) were obtained for wild type strain *Y. lipolytica* A-101 and its acetate negative mutants growing in the medium with glycerol (Rywińska et al., 2010; Rymowicz et al., 2010).

The biosynthesis of CA and invertase carried out in the medium with different carbon sources provided distinct amount of biomass as waste byproduct (Table 2). The highest amount of *Y. lipolytica* A-101-B56 biomass was obtained from sucrose as a carbon source, while the lowest from glycerol (12.4 and 5.8 g/L, respectively), although glucose, fructose and glycerol are catabolized through the same biochemical pathways. In spite of the initial concentrations of substrates were comparable, the fermentation media prepared on their basis differed in osmolality (see Materials and Methods). So, the highest amount of biomass obtained in the sucrose based medium was probably due to the lowest osmotic pressure, while the highest osmolality of glycerol medium slowed yeast growth. Similar differences in biomass accumulation were observed for other *Y. lipolytica* strains growing from glucose and/or glycerol (Rywińska et al., 2010). Although in the medium with mixture of both substrates, glycerol was consumed before glucose.

The CA biosynthesis in sucrose based media performed with other *SUC*$^+$ clones of *Y. lipolytica* provided comparable or slightly higher (up to 16.9 g/L) amount of biomass (Wojtatowicz et al., 1997; Förster et al., 2007). Similar or higher amount of biomass (up to 21 g/L) produced wild type strain *Y. lipolytica* A-101 and its mutants using pure or crude glycerol as a carbon source (Rywińska et al., 2010). Some CA biosynthesis processes are compared in Table 3.

Another important parameters of the simultaneous biosynthesis of CA and invertase are the level and order of substrate utilization (Fig. 2). Throughout BC, the transformant *Y. lipolytica* A-101-B56-5 utilized glucose and glycerol with constant consumption rates of 1.46 and 1.55 g/L/h, respectively. Sucrose consumption rate was changing. Initially, cells hydrolyzed sucrose very slowly but after about 12 h rapidly, with a very high rate of 5.45 g/L/h. After 26 h of culture sucrose was completely hydrolysed. Slower rate of sucrose hydrolysis (up to 40–46 h) presented *SUC*$^+$ transformants of *Y. lipolytica* studied by other scientists (Förster et al., 2007; Wojtatowicz et al., 1997). Only, the strain *Y. lipolytica* W29-302 ura3 hydrolyzed sucrose rapidly, in 27 h (Żarowska et al., 2001). Yeast cells of *Y. lipolytica* A-101-B56-5 clone preferably used glucose and at the time when the medium lacked this carbon source, cells began to utilize fructose. This trend was clearly observed for this transformant in the medium with mixture of glucose and fructose as substrates. Preference of glucose consumption over fructose for life processes was also observed by others (Wojtatowicz et al., 1997; Żarowska et al., 2001; Förster et al., 2007).

Noticeable production of invertase by *Y. lipolytica* A-101-B56-5 began at around 8–10 h of BC for extracellular enzyme and from

Table 3
Parameters of CA biosynthesis by *Y. lipolytica* strains from different substrates.

Y. lipolytica strain	Substrate	X (g/L)	CA (g/L)	$Y_{CA/S}$ (g/g)	Q_{CA} (g/L/h)	q_{CA} (g/g/h)	References
H222	Glucose	23.70	100.00	0.65	1.140	0.050	Moeller et al. (2010)
H222	Glucose	12.50	41.00	0.55	0.450	0.041	Moeller et al. (2007)
W29	Glucose	5.00	49.00	0.85	0.180	0.036[a]	Papanikolaou et al. (2009)
ACA-DC 50109	Glucose + olive-mill wastewater	7.76	28.90	0.53	–	–	Papanikolaou et al. (2008)
A-101.1.31	Glucose + sodium acetate	11.30	58.00	0.85	1.020	0.080	Robak et al. (2007)
AWG7	Glycerol	11.00	82.90	0.53	0.660	0.060	Rywińska et al. (2010)
ACA-DC 50109	Glycerol	5.92	33.55	0.52	–	–	Makri et al. (2010)
A-101.1.22	Crude glycerol	17.00	124.20	0.77	0.850	0.050	Rymowicz et al. (2010)
ACA-YC 5033	Crude glycerol	6.50	50.10	0.44	–	–	André et al. (2009)
VKM Y-2373	Ethanol	11.72	105.40	0.88	1.225	0.138	Arzumanov et al. (2000)
N1	Ethanol	~7	120.00	0.87	1.150	0.120	Kamzolova et al. (2003)
DSM 3286	Olive oil	6.66	3.60	0.393	0.054	0.008	Darvishi et al. (2009)
H222-S4 T5	Sucrose	8.00	140.00	0.82	0.730	0.091	Förster et al. (2007)
A-101-B56-5	Sucrose	12.40	45.02	0.64	0.625	0.050	This study
A-101-B56-5	Glycerol	5.80	57.15	0.60	0.794	0.137	This study

[a] Calculated according to presented data.

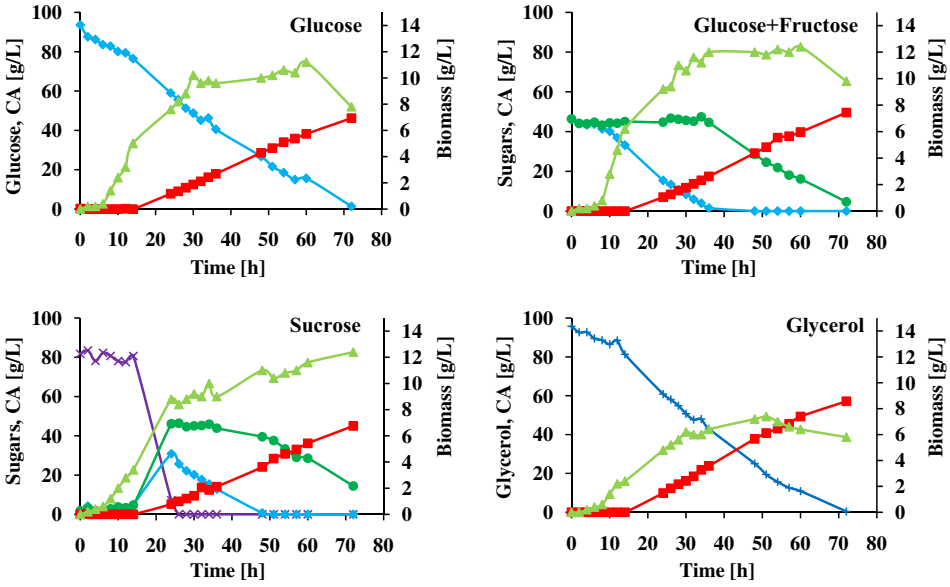

Fig. 2. Concentration of glucose (♦), fructose (●), sucrose (×), glycerol (+), CA (■) and biomass (▲) during citrate accumulation by *Y. lipolytica* A-101-B56-5 from different substrates.

about 24 h for intracellular one (Fig. 3). Yeast cells secreted invertase with average rate ranging from 82 to 132 U/L/h and intracellularly accumulated it with average rate of 1000–1356 U/L/h. The highest extracellular and intracellular invertase activity was detected in sucrose medium, 3720 and 69 130 U/L, respectively (Table 1 and Table 4).

The natural yeast isolates of *S. cerevisiae,* the most frequently used microorganisms for the production of invertase, in sucrose based medium produced about 1900 U of enzyme in 1 liter of culture (Ul-Haq et al., 2008). Genetic modifications of these strains improved their capacity for invertase biosynthesis, which resulted in an enzyme preparation with activity over 58 000 U/L. There are also known hyperproducing strains of *S. cerevisiae,* which produced up to 120 000 U/L of invertase (Ul-Haq and Ali, 2005). Another fungi species Xanthophyllomyces dendrorhous and Thamnidium elegans growing in sucrose medium secreted 3500 and 1400 U/L of invertase, respectively (Linde et al., 2009; Papanikolaou et al., 2010). Also, invertase biosynthesis in solid state fermentation using filamentous fungi *A. niger* was not competitive process to the one presented in this manuscript. The obtained enzyme activity was only 300 U/L of culture (Aranda et al., 2006). Comparison of some invertase producers was presented in Table 5.

Yield of extra- and intracellular invertase biosynthesis from tested substrates by *Y. lipolytica* A-101-B56-5 was similar independently of carbon source (Table 2). The highest value of this parameter for extracellular enzyme was 46 U/g and for intracellular one 455 U/g of sucrose. It is worth to mention that the results of invertase yield presented by others were much lower than the values obtained in this study. Recombined *SUC*$^+$ *Y. lipolytica* H222-S4 T5 strain produced only 0.62 U of invertase from 1 g of sucrose (Förster et al., 2007) and hyperproducing strains of *S. cerevisiae*approximately only 6 U from 1 g of that substrate (Ul-Haq and Ali, 2005; Ul-Haq et al., 2008).

What is more, very high results were obtained in this study, concerning invertase activity units per gram of yeast biomass of *Y. lipolytica* A-101-B56-5, accumulated on different substrates (Table 2). Invertase yield from biomass ranged from 2568 to 3736 U/g of cells for intracellular enzyme and from 232 to 589 U/g for extracellular one. In the last case, one of the highest recorded values was reached. For another *SUC*$^+$ strain of *Y. lipolytica* the yield of invertase from biomass was 105 U/g (Förster et al., 2007). Hyperproducing strains of *S. cerevisiae* and their mutants also produced invertase with lower yield, ranging from 60 to 120 U/g of biomass (Ul-Haq and Ali, 2005; Ul-Haq et al., 2008). Also, lower results of yield, were obtained for solid-state fermentation with filamentous

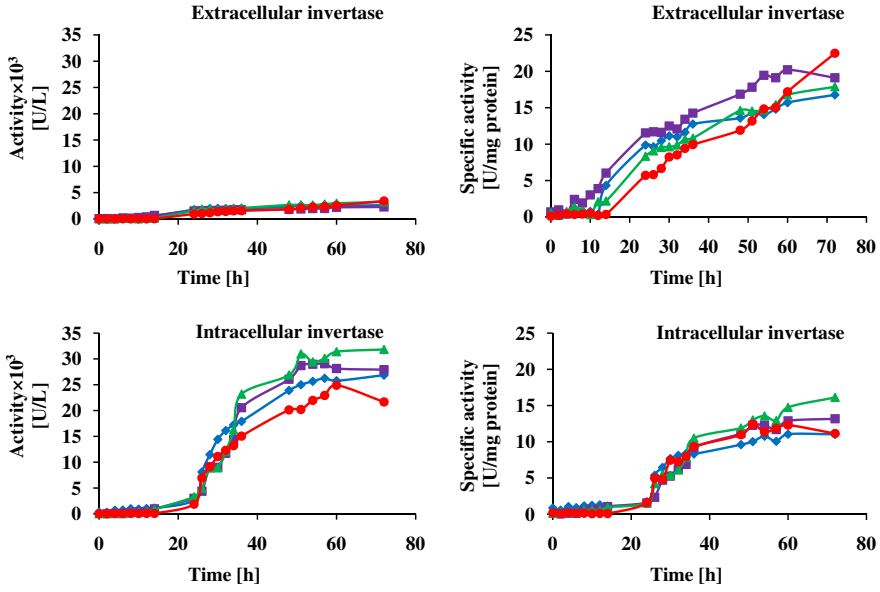

Fig. 3. Invertase activity during citrate fermentation using *Y. lipolytica* A-101-B56-5 growing in medium with different substrates (glucose ♦, glucose + fructose ■, sucrose ▲, glycerol ●).

Table 4
Invertase activity and protein content in *Y. lipolytica* A-101-B56-5 post-culture media, cell's extracts (baking soda) and their concentrates.

Substrate	Type of sample	Protein concentration (mg/L)	Activity (U/L)	Specific activity (U/mg protein)
Extracellular invertase				
Glucose	Post culture medium	160 ± 10	2599 ± 93	16.2 ± 0.3
	Concentrate	940 ± 50	132 500 ± 9275	141 ± 2.8
Glucose + Fructose	Post culture medium	200 ± 10	2273 ± 102	11.4 ± 0.04
	Concentrate	900 ± 50	119 800 ± 7787	133 ± 1.3
Sucrose	Post culture medium	180 ± 10	3253 ± 88	18.1 ± 0.7
	Concentrate	5030 ± 230	484 900 ± 34 913	96.0 ± 2.6
Glycerol	Post culture medium	150 ± 10	3416 ± 232	22.8 ± 0.1
	Concentrate	2000 ± 100	188 100 ± 15 048	94.0 ± 3.0
Intracellular invertase				
Glucose	Cell's extract	1850 ± 120	4040 ± 392	2.2 ± 0.1
	Concentrate	2820 ± 230	125 400 ± 6772	44.0 ± 1.3
Glucose + Fructose	Cell's extract	2170 ± 180	17 100 ± 958	7.9 ± 0.2
	Concentrate	3130 ± 250	226 980 ± 20 428	73.0 ± 0.8
Sucrose	Cell's extract	2350 ± 180	69 130 ± 4977	29.4 ± 0.1
	Concentrate	4510 ± 280	1849 210 ± 88 762	410 ± 6.2
Glycerol	Cell's extract	2200 ± 110	22 300 ± 1427	10.1 ± 0.2
	Concentrate	4720 ± 270	454 760 ± 27 740	96.0 ± 0.4

Table 5
Comparison of some invertase producers.

Invertase producer	Yield from biomass (U/g)	Activity (U/L)	References
A. niger Aa20	72	300	Aranda et al. (2006)
A. niger	4000	840	Rubio and Navarro (2006)
T. elegans CCF-1465	128	1400	Papanikolaou et al. (2010)
S. cerevisiae	540	965 000	Hustedt et al. (1990)
S. cerevisiae GCA-II (hyperproducer)	121	107 400	Ul-Haq and Ali (2005)
R. glutinis CIM 1001	–	384	Rubio et al. (2002)
X. dendrorhous ATCC MYA-131	–	3500	Linde et al. (2009)
Y. lipolytica H222-S4 T5	105	840	Förster et al. (2007)
Y. lipolytica A-101-B56-5	2568	69 130	This study
(on sucrose)	149 129[a]	1 849 210[a]	
Y. lipolytica A-101-B56-5	3736	22 300	This study
(on glycerol)	78406[a]	454 760[a]	

[a] Concentrates of invertase.

fungi *A. niger* (Aranda et al., 2006). Results of invertase yield (540 U/g) presented by Hustedt et al. (1990) for *S. cerevisiae* based commercial method of enzyme production (mechanical pulping of previously obtained yeast biomass) were comparable to invertase yield from biomass of *Y. lipolytica* A-101-B56-5. Only for *A. niger* growing from inulin higher yield of invertase, amounting to 4000 U from 1 g of biomass was obtained (Rubio and Navarro, 2006).

Intracellular invertase process productivity obtained with strain *Y. lipolytica* A-101-B56-5 ranged from 300 to 442 U/L/h (Table 2). More than 4 times higher results of productivity (2238 U/L/h) was shown for hyperproducing strain of *S. cerevisiae* (Ul-Haq and Ali, 2005). However, taking into consideration the specific production rate of invertase by cells of *Y. lipolytica* A-101-B56-5, the obtained values (35.6–51.8 U/g/h) were significantly higher compared to specific production rate (2.5 U/g/h) of hyperproducing strain of *S. cerevisiae* (Ul-Haq and Ali, 2005). Results of this parameter obtained for *Y. lipolytica* A-101-B56-5 were comparable to the results obtained by Aranda et al. (2006) for solid-state fermentation using *A. niger* (34.3 U/g/h). Ultrafiltration process, aimed at pre-concentration and purification of invertase, led to significant increase in enzyme activity (Table 4). The resulting enzymes concentrates possessed an extracellular invertase activity from 119 800 to 484 900 U/L, with specific activity 94–141 U/mg of protein. In the case of intracellular enzyme very high increase in its activity was observed. The values of this parameter ranged from 125 400 up to 1 849 210 U/L, with specific activity 44–410 U/mg of protein. The highest activity value was determined for invertase extracted from biomass growing from sucrose.

4. Conclusions

Summarizing the study of simultaneous biosynthesis of CA and invertase, the strain *Y. lipolytica* A-101-B56-5 was a very good producer of both metabolites. Using this strain high concentration of CA as well as intra- and extracellular invertase with a very high yield were achieved. The waste of biomass after extraction of intracellular invertase using an inexpensive method with baking soda can be used as feed supplement. This process can successfully be applied in industry due to the fact that it is a waste-free and therefore cost-effective and environmentally friendly one.

Acknowledgements

Authors would like to thank Prof. Gerold Barth and Dr. Stephan Mauersberger from Institute of Microbiology, University of Technology, Dresden, Germany for plasmids and help in yeast transformation as well as MD. Piotr Dziunycz from Department of Dermatology, University Hospital of Zurich, Switzerland for help

in molecular biology techniques and Prof. Waldemar Rymowicz from Department of Biotechnology and Food Microbiology, Wroclaw University of Environmental and Life Sciences for the access to bioreactors.

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