

# Characterization of a S-adenosyl-L-methionine (SAM)-accumulating strain of *Scheffersomyces stipitis*

Stela Križanović,<sup>1</sup> Ana Butorac,<sup>2</sup> Jasna Mrvčić,<sup>1</sup> Maja Krpan,<sup>1</sup> Mario Cindrić,<sup>3</sup>  
Višnja Bačun-Družina,<sup>2</sup> Damir Stanzer<sup>1\*</sup>

<sup>1</sup>Laboratory for Fermentation and Yeast Technology, Faculty of Food Technology and Biotechnology, University of Zagreb, Zagreb, Croatia. <sup>2</sup>Laboratory for Biology and Microbial Genetics, Faculty of Food Technology and Biotechnology, University of Zagreb, Zagreb, Croatia. <sup>3</sup>Laboratory for System Biomedicine and Centre for Proteomics and Mass Spectrometry, Ruder Bošković Institute, Zagreb, Croatia

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**Summary.** S-adenosyl-L-methionine (SAM) is an important molecule in the cellular metabolism of mammals. In this study, we examined several of the physiological characteristics of a SAM-accumulating strain of the yeast *Scheffersomyces stipitis* (M12), including SAM production, ergosterol content, and ethanol tolerance. *S. stipitis* M12 accumulated up to 52.48 mg SAM/g dry cell weight. Proteome analyses showed that the disruption of C-24 methylation in ergosterol biosynthesis, a step mediated by C-24 sterol methyltransferase (Erg6p), results in greater SAM accumulation by *S. stipitis* M12 compared to the wild-type strain. A comparative proteome-wide analysis identified 25 proteins that were differentially expressed by *S. stipitis* M12. These proteins are involved in ribosome biogenesis, translation, the stress response, ubiquitin-dependent catabolic processes, the cell cycle, ethanol tolerance, posttranslational modification, peroxisomal membrane stability, epigenetic regulation, the actin cytoskeleton and cell morphology, iron and copper homeostasis, cell signaling, and energy metabolism. [Int Microbiol 2015; 18(2):117-125]

**Keywords:** *Scheffersomyces stipitis* · S-adenosyl-L-methionine (SAM) · SAM accumulating yeast · C-24 sterol methyltransferase (Erg6p)

## Introduction

S-adenosyl-L-methionine (SAM, also known as AdoMet) is a catalytic and synthetic cofactor in enzymatic reactions and participates in many biological processes [21], including as a methyl group donor for methyltransferase reactions in the biosynthesis of nucleic acids, proteins, phospholipids, and

sterols [20]. SAM has found wide application in medicine as a chemotherapeutic agent in the treatment of a broad range of diseases, including depression, liver disease, Lesch-Nyhan disease, Alzheimer's disease, and diarrhea [26]. These promising therapeutic results have increased the demand for SAM.

The yeasts *Saccharomyces cerevisiae*, *Saccharomyces sake*, *Pichia pastoris*, *Saccharomyces uvarum*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, and *Candida* species have been studied for their SAM-producing ability [5,16,24]. *Scheffersomyces stipitis*, formerly known as *Pichia stipitis* [14], is a Crabtree-negative yeast able to grow on different carbon, nitrogen, sulfur, and phosphorus sources [3]. This growth flexibility makes it a viable economic candidate for the industrial production of a variety of value-added products, such as SAM.

\*Corresponding author: D. Stanzer  
Faculty of Food Technology and Biotechnology  
University of Zagreb  
Pierottijeva 6  
10000 Zagreb, Croatia  
Tel. + 385-14605286. Fax +385-14605072  
E-mail: dstanzer@pbf.hr

Ergosterol biosynthesis is one of many metabolic pathways in which SAM is required [20]. Ergosterol, the major sterol in yeast [8], participates in numerous structural and signaling functions, including membrane permeability, membrane fluidity, the activity and distribution of integral proteins, and cell cycle control [7]. Most of the more than 20 proteins that contribute to ergosterol biosynthesis are essential; only five proteins, involved in the final steps of the pathway, are nonessential [8]. Among the latter is C-24 sterol methyltransferase (Erg6p), which catalyzes a late step of ergosterol biosynthesis [27] in which SAM acts as the methyl group donor. In previous studies, strains of *S. cerevisiae* and *Candida* sp. defective in ergosterol biosynthesis were isolated and characterized [7,16,18,24]. In the present work, we characterized a SAM-accumulating strain of *S. stipitis* (M12) with respect to SAM production, ergosterol content, and ethanol tolerance, and also analyzed its proteomic profile.

## Materials and methods

**Strains and cultivation media.** Wild-type *Scheffersomyces stipitis* BS 5776 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). A SAM-accumulating strain of *S. stipitis*, M12, was obtained by UV irradiating strain BS 5776 at a dose of 160 J/m<sup>2</sup> and subsequent isolation on YPD medium (1% yeast extract, 2% peptone, 2% glucose) containing 15 µg nystatin/ml [13]. YPD medium was used for yeast cultivation and YPDE medium [YPD with 0.5–5% (v/v) ethanol] for ethanol-tolerance testing [11]. The additive effect of D,L-methionine on SAM production was determined by cultivating the yeast on modified O-medium (5% D-glucose, 1% peptone, 0.6% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5% yeast extract, 0.4% KH<sub>2</sub>PO<sub>4</sub>, 0.2% K<sub>2</sub>HPO<sub>4</sub>, and 0.05% MgSO<sub>4</sub> · 7 H<sub>2</sub>O, pH 6) containing 0.6% D,L-methionine [24]. The cells were cultivated in Erlenmeyer flasks placed on a shaker (200 rpm) at 30°C for 48 h. Stationary-phase cells of *S. stipitis* strain M12 and of *S. stipitis* wild-type were harvested by centrifugation at 4000 ×g for 10 min, washed twice with sterilized distilled water, and then analyzed as described below. Growth was measured gravimetrically and expressed as grams of cell dry weight (CDW) per liter.

**Determination of SAM and ergosterol in yeast cells.** SAM production was quantified using the HPLC method of Valko et al. [29]. The

amount of SAM in the extracted supernatant was analyzed on a ChromSep HPLC SS column (250 × 4.6 mm) using an IonoSpher 5C guard column with the Varian Prostar 230 pumping system and a UV lamp at 260 nm. Yeast sterols were extracted and the ergosterol content was measured following the procedure described by Arthington-Skaggs et al. [1].

**Isolation of lipid particles and microsomal fractions.** Lipid particles and microsomal protein fractions were isolated as described by Mo et al. [17]. The protein content in the isolated fractions was measured using a Bradford assay.

**SDS-PAGE and protein identification by MALDI TOF/TOF mass spectrometry.** The microsomal protein fraction was separated by 1-D SDS-PAGE (10% polyacrylamide gel) and then visualized by Coomassie blue staining [15]. Protein bands with a molecular mass of 35–50 kDa were excised from the gel and subjected to tryptic in-gel digestion as described by Shevchenko et al. [23]. After digestion, the extracted peptides were purified on C<sub>18</sub> ZipTip columns and evaporated to dryness in a SpeedVac. The dried samples were dissolved in 5 µl of α-cyano-4-hydroxycinnamic acid (5 mg/ml CHCA matrix dissolved in water/MeCN mixture, 1:1 v/v) and spotted onto a metal matrix-assisted laser desorption/ionization (MALDI) plate. Mass spectrometry (MS) was performed using a MALDI time of flight (TOF)/TOF 4800 Plus tandem mass spectrometer (Applied Biosystems) equipped with a 200-Hz, 355-nm Nd: YAG laser. Acquisitions were performed in positive ion reflectron mode. Mass spectra were obtained by averaging 1800 laser shots covering a mass range *m/z* 800–4000. Internal calibration of the mass range was performed with tryptic autolysis fragments. Sterol methyltransferase was digested in silico to generate a list of peptide ions suitable for further MS/MS analyses (maximum of two trypsin miscleavages). MS/MS analysis was completed under a 1-kV collision energy in positive ion mode with air used as the collision gas. Protein identification and database searching were performed by GPS Explorer Software v3.6 (Applied Biosystems). The results of the combined ion searches using MS and MS/MS data were matched against the NCBIInr using the MASCOT search engine [19]. The parameters were two missed trypsin cleavages and oxidation on methionine within a mass tolerance of 21 ppm. Protein scores > 71 were considered as significant (*P* < 0.05).

**DNA isolation and ERG6 gene sequencing.** Chromosomal DNA was prepared using the protocol for yeast *Saccharomyces cerevisiae* [2]. Polymerase chain reactions (PCRs) were carried out using Hot Start Taq DNA polymerase. The *ERG6* gene was sequenced with specific primers (Sigma Aldrich). Both the primer sequences and the annealing temperatures are shown in Table 1. PCR amplification was performed with an Eppendorf Mastercycler EP. PCR-amplified DNA was analyzed by gel electrophoresis (40 min at 8.3 V/cm) on a 1% agarose gel in TBE buffer. The amplified products were purified from the gel using the QIAquick gel extraction kit. Sequencing was carried out on an ABI PRISM 3100-Avant genetic analyzer (Applied

**Table 1.** Sequences and conditions of oligonucleotide primers used in PCR analysis

Scheffersomyces stipitis gene name (gene product)	GenBank accession no.	Primer	Nucleotide sequence (5' to 3')	Nucleotide coordinates*	Conditions
ERG6 (Erg6p, C-24 sterol methyltransferase)	gi126257970	ERG6-1F	GTTCACCGGGCTTCAAAAC	2481588 to 2481607	1× at 95°C for 4 min
		ERG6-1R	AGCCTGGACACCAAAAGTCT	2482401 to 2482382	1× at 59°C for 30s
		ERG6-2F	AGGTCATGTGCAACAGTTCAGT	2480818 to 2480839	35× at 72°C for 50s
		ERG6-2R	GTTTTGAAGCCCGGTGGAAC	2481607 to 2481588	1× at 72°C for 7min
		ERG6-3F	AGCCAGAGACCTTTGATGCC	2480596 to 2480615	
		ERG6-3R	TGCTTAGAGCCCTTTGGAGC	2481052 to 2481033	

\*Nucleotide coordinates refer to the corresponding gene sequence in the GenBank database.

Biosystems) using the same primers as in the genomic DNA amplification. DNA was verified in both the sense and antisense directions. The sequence was submitted to GenBank with the accession number KR01466.

#### Sample preparation for comparative proteomic analysis.

The cells were disrupted and lysed with 0.5 mm glass beads at 4°C, followed by centrifugation at 3000 ×g for 10 min at 4°C. The isolated cytosolic proteins were subjected to tryptic digestion (final trypsin concentration of 20 µg/ml) for 18 h at 37°C.

#### Ultra performance liquid chromatography (UPLC)–MS<sup>E</sup> in the analysis of cytosolic proteins.

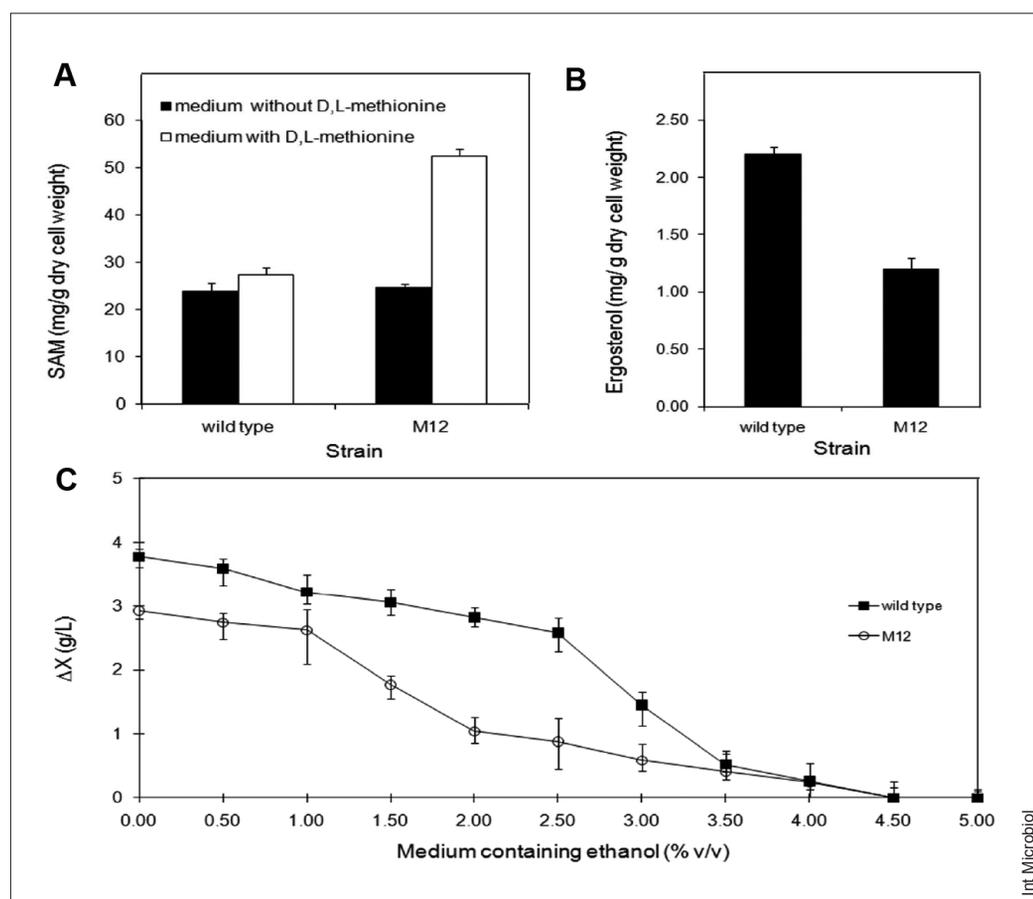
Peptide samples were analyzed by nano-UPLC-MS<sup>E</sup> using an Acquity UPLC column BEH130 C<sub>18</sub> (100 µm × 100 mm; Waters, Milford) and a 60-min gradient of 0.1–99% solvent B (solvent A: 99.9% H<sub>2</sub>O, 0.1% formic acid; solvent B: 95% acetonitrile, 0.1% formic acid in H<sub>2</sub>O) on a Waters nanoAcquity UPLC system (flow rate 1 µl/min) coupled to an ESI-qTOF SYNAPT G2-Si (resolution mode of operation) mass spectrometer (Waters). A pre-column 2G-V/M 5 µm Symmetry C18 trap (180 µm × 20 mm), with a flow rate of 15 µl/min, was used to desalt the samples prior to their separation. LC-MS data were collected in alternating low-energy and elevated-energy (MS<sup>E</sup>) modes of acquisition. The variables were as follows: positive ion mode, desolvation nitrogen flow 0.6 bar at 150°C, capillary voltage of 3.5 kV, and a cone voltage of 40 V. The spectral acquisition time in each mode was 1 s. In low-energy MS mode, data were

collected at constant collision energy of 4 eV. In elevated-energy MS mode, the collision energy was ramped from 20 to 45 eV during each 1-s data collection cycle. Each sample was analyzed in triplicate runs. The mass accuracy of the raw data was corrected using leucine enkephalin (1 ng/µl, 0.4 µl/min flow rate, 556.2771 Da [M + H]<sup>+</sup>), which was infused into a mass spectrometer as a lock mass during sample analysis. The raw data were processed with a ProteinLynx Global Server (PLGS; version 3.0.1; Waters). The UniProt *S. stipitis* database (release 2015\_01, January 2015, 5570 entries) was used for database searches with the following parameters: peptide tolerance 10 ppm, fragment tolerance 0.015 Da, trypsin-missed cleavages 2, and methionine oxidation.

**Statistical analysis.** The data are presented as the means ± SD of three independent experiments.

## Results

**SAM production.** SAM is formed in yeast cells from D,L-methionine and ATP [5]. The effect on the SAM content of *S. stipitis* strain M12 grown in medium containing 0.6% D,L-methionine was therefore determined (Fig. 1A). In medium



**Fig. 1.** (A) SAM and (B) ergosterol contents of *Scheffersomyces stipitis* strain M12 and *S. stipitis* wild-type. (C) Effect of ethanol on the growth of *S. stipitis* M12 strain and *S. stipitis* wild-type in YPD (without ethanol) and YPDE (with 0.5–5% ethanol) media. The values are the means ± SD of three independent measurements.

without D,L-methionine, the SAM content of strain M12 (24.7 mg/g CDW) was similar to that of the wild-type strain (23.95 mg/g CDW). The addition of D,L-methionine to the medium increased the content of SAM in both strains, but SAM accumulation in the M12 strain (52.48 mg/g CDW) was two-fold higher than in the wild-type strain (26.37 mg/g CDW).

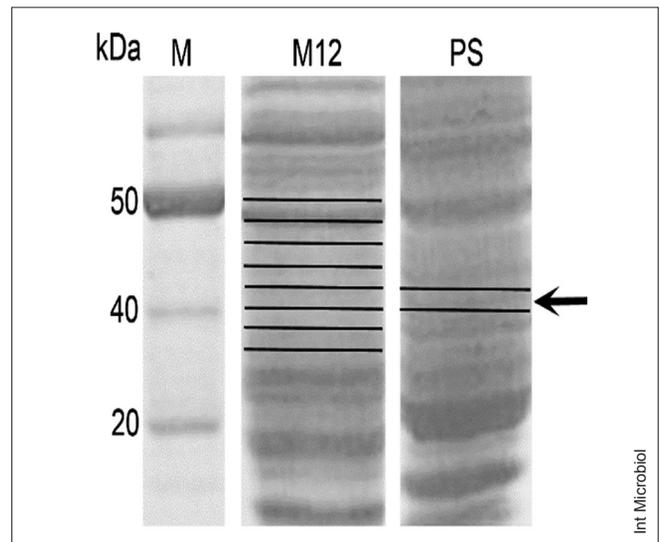
**Ergosterol yield.** SAM accumulates in yeast strains defective in ergosterol biosynthesis [24]. We therefore measured the ergosterol content in the wild-type and SAM accumulating strain of *S. stipitis* (Fig. 1B). The ergosterol content was two-fold lower in strain M12 (1.1 mg/g CDW) than in the wild-type (2.2 mg/g CDW) (Fig. 1B).

### The effect of ethanol on *Scheffersomyces stipitis* growth.

Interruption of the ergosterol biosynthesis pathway in yeast can result in physiological changes in the membrane that affect ethanol tolerance [11]. An analysis of the effect of ethanol on the growth of *S. stipitis* wild-type and strain M12 was carried out using medium without ethanol (YPD) and media containing various amounts of ethanol [YPDE; 0.5–5 % ethanol] (Fig. 1C). The cell yield in YPD medium was 3.00 g CDW/l for strain M12 and 3.78 g CDW/l for the wild-type. In YPDE medium the total cell yield of the wild-type decreased significantly when the yeast was cultured in medium containing 3% ethanol (1.45 g CDW/l); however, for strain M12 the decrease in total cell yield was already significant in medium containing 1.5% ethanol (1.77 g CDW/l). In YPDE medium containing > 3% ethanol, the growth of M12 was nearly the same as that of the wild-type.

### *Scheffersomyces stipitis* Erg6p protein analysis.

To the best of our knowledge, the expression of Erg6p in *S. stipitis* has not been reported. Using the NCBI database ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)), we detected a protein similar to *S. cerevisiae* S288c Erg6p. The accession number of the *S. cerevisiae* Erg6p protein in the NCBI database is 6323635 [4]. Using the BLASTp algorithm [[www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)], we matched *S. cerevisiae* Erg6p against the *S. stipitis* CBS 6054 annotated genome and found an analog protein, denoted as a “predicted protein,” with 95% similarity to *S. cerevisiae* S288c Erg6p. The analog protein (accession number 126275196) contains 377 amino acids and has a molecular mass of 43,323 Da. The *S. cerevisiae* protein has 383 amino acids and a molecular mass of 43,431 Da. To confirm the cellular expression of Erg6p in *S. stipitis*, the microsomal fraction of the yeast cells was isolated and separated by one-dimensional (1-DE) SDS-PAGE (Fig. 2). Protein bands with a

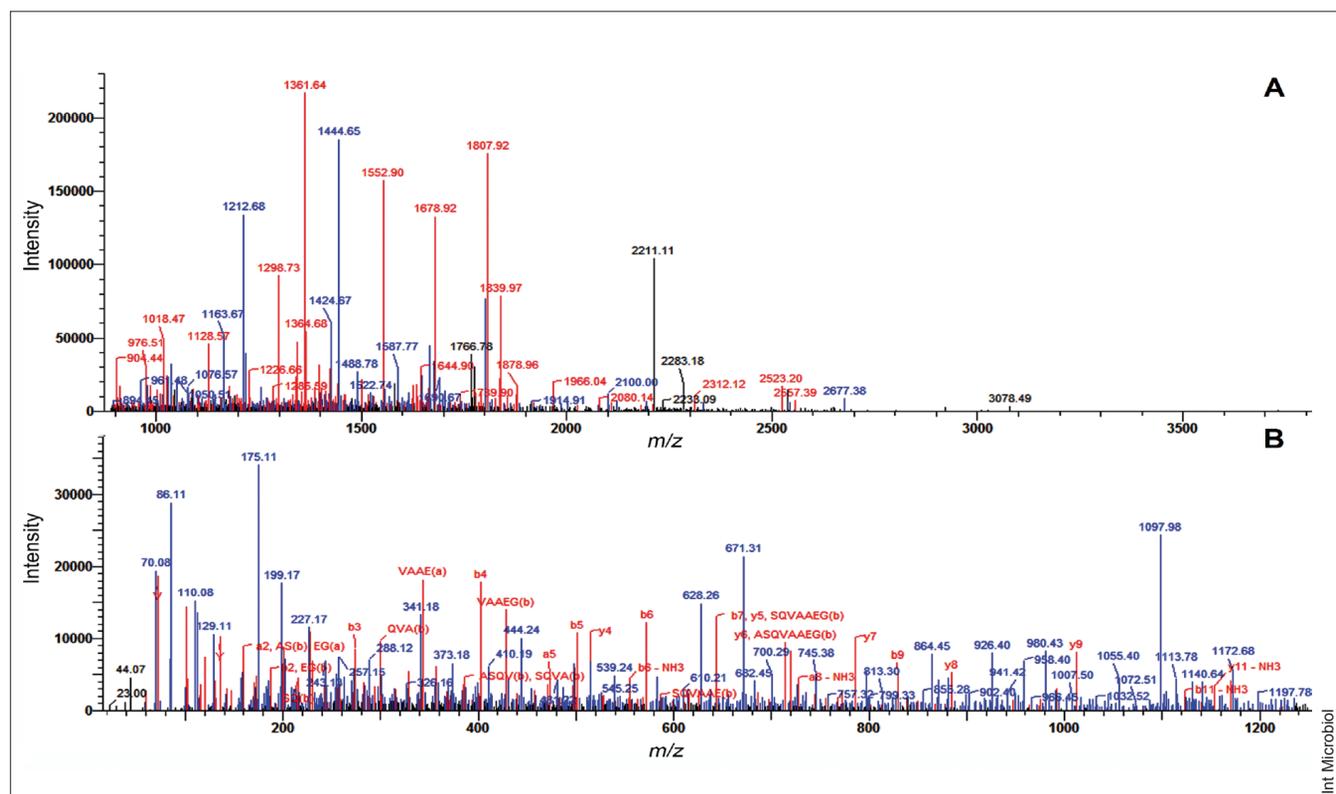


**Fig. 2.** SDS-PAGE of the microsomal fraction of proteins extracted from *Scheffersomyces stipitis* M12 strain and *S. stipitis* wild-type. Proteins with a molecular mass of 35–50 kDa were excised from the gel and analyzed by mass spectrometry. Erg6p was identified in protein bands excised from *S. stipitis* wild-type (marked with an arrow and black border) but was not detected within a range of 35–50 kDa in strain M12.

molecular mass of 35–50 kDa were subjected to tryptic in-gel digestion. The isolated proteins were identified by a MALDI-TOF/TOF MS/MS and a database search (Fig. 3). The results confirmed the expression of the “predicted” protein and that it belongs to the Erg6p family. The microsomal proteins from *S. stipitis* M12 strain and the wild-type were isolated and then separated by 1-DE SDS-PAGE (Fig. 2). Erg6p was positively confirmed by mass spectrometry in the wild-type (Fig. 3) but was not detected in *S. stipitis* M12 strain, neither at the same excision spot nor in the mass range of 35–50 kDa. These results suggested that Erg6p is not expressed by strain M12.

### Comparative proteomic analysis of *Scheffersomyces stipitis* strain M12 and the wild-type.

The cytosolic protein fraction from the wild-type and strain M12 strain was subjected to a comparative proteomic analysis by UPLC-MS<sup>E</sup>. A comparison of the proteomic profiles of the wild-type and strain M12 showed a difference of 25 proteins (Table 2). Proteins overexpressed in strain M12 relative to the wild-type were involved in ribosome biogenesis, translation, the stress response, and ubiquitin-dependent catabolic processes, whereas proteins down-regulated in strain M12 compared to the wild-type were involved in the cell cycle, translation, the stress response, ethanol tolerance, posttranslational modification, protein-protein interactions, the catalytic removal of an amino group, peroxisomal membrane stability,



**Fig. 3.** (A) MALDI-TOF mass spectra of tryptic peptides extracted from the gel (43 kDa band). (B) MS/MS spectrum of the peptide ion  $m/z$  1285.5933 obtained in CID mode. The protein identified against the MASCOT search engine is Erg6p. Red peaks indicate matched peaks.

metal ion transport, epigenetic regulation, the membrane targeting of proteins, the actin cytoskeleton and cell morphology, translational elongation, iron and copper homeostasis, cell signaling, and energy metabolism.

Based on these differentially expressed proteins, we mapped the protein-protein interactions using the STRING web-tool and the UniProt *S. stipitis* database (Fig. 4). Two separate groups of proteins were thus identified: interacting proteins and non-interacting proteins. The interacting proteins were separated into two networks. The first consisted of ten proteins, nine belonging to *S. stipitis* wild-type and one belonging to *S. stipitis* strain M12. All of these proteins are involved in protein synthesis and processing. In this network, ribosomal protein L16b/L23e (PICST\_89371), 60s ribosomal protein L13 (RPL13), and ribosomal protein S7A (RPS7A) interacted with 7 proteins (out of 10); a predicted protein (PICST\_76243) interacted with 6 proteins; the 40s ribosomal protein (PICST\_85487), elongation factor 1- $\alpha$  (TEF1), and ribosomal protein L37 (PICST\_76246) interacted with 5 proteins; signal recognition particle subunit (SEC65) interacted with 3 proteins; and two predicted protein (PICST\_39616 and PICST\_39616) interacted with 1 protein each. The second

network consisted of only two proteins, one belonging to *S. stipitis* M12 strain and the other to *S. stipitis* wild-type. These proteins are associated with mitochondria.

## Discussion

Several strains and species of yeasts have been screened for SAM production [5,16,24]. To increase SAM accumulation in *S. stipitis* CBS 5776, we produced a SAM-accumulating strain (M12) with interrupted ergosterol biosynthesis [13]. In the biosynthesis of ergosterol, C-24 sterol methyltransferase (Erg6p) mediates the transfer of a methyl group from SAM to zymosterol, forming fecosterol. This step is metabolically expensive for the cell, requiring 12–14 ATP equivalents [27]. Blocking this reaction could enhance SAM accumulation in two ways, by preventing the yeast cells from spending the accumulated SAM or by costing them less ATP in the biosynthesis of ergosterol; this ATP could then be used in SAM biosynthesis [24]. Together with ATP, D,L-methionine is required for SAM biosynthesis [5]. We showed that the addition of D,L-methionine to the medium stimulated SAM production by

**Table 2.** Overview of the proteins that are differentially expressed between *S. stipitidis* M12 strain and *S. stipitidis* wild type

Accession no <sup>a</sup>	Protein name	Gene	MW <sup>b</sup> (kDa)	pI <sup>c</sup>	Coverage (%)	Precursor Mass Error (ppm)	Expression	Function
A3LUM4	Predicted protein	PICST_31817	15.03	5.04	13.43	5.67	+	Mitochondrial precursor, ribosome biogenesis
A3LZU1	40S ribosomal protein	PICST_85487	15.92	9.94	19.44	2.83	+	Translation
A3LPX4	Predicted protein	PICST_67049	19.57	5.98	17.18	6.79	+	Response to stress
A3LWU4	Predicted protein	PICST_32591	18.90	4.32	29.87	6.23	+	Ubiquitin-dependent protein catabolic process
A3LT69	Ribosomal protein L14b/L23e	PICST_89571	14.47	10.65	14.59	7.76	-	Translation
A3LZ15	Heat shock protein of the HSP70 family (SSB1)	HSP70.1	66.40	5.06	7.34	7.25	-	Response to stress
A3LVA1	Suppressor of lethality protein	SHP1	40.70	4.65	14.79	4.32	-	Cell cycle, DNA processing, tolerance to ethanol
A3LNK8	Predicted protein	PICST_56381	10.97	4.97	9.37	4.47	-	Posttranslational modification of protein
A3M0L8	Ankyrin repeat protein	ANK2	19.98	4.18	36.81	6.02	-	Mediate innumerable protein-protein interaction
A3GFL1	Maintenance of mitochondrial DNA	MIMD1	12.02	5.52	19.26	3.39	-	Catalysis of the removal of an amino group from substrate
A3LP14	Ribosomal protein S7A	RPS7A	21.26	10.25	13.82	2.22	-	Translation
A3LPL6	Hypothetical protein	PICST_29989	70.90	4.62	9.72	6.58	-	Unknown
A3LZ32	40kDa farnesylated protein associated with peroxisomes	PEX19	35.84	4.00	29.60	5.62	-	Required for proper localization and stability of peroxisomal membrane proteins, tolerance to ethanol
A3LY98	Signal recognition particle subunit	SEC65	31.27	6.78	30.07	3.10	-	SRP-dependent cotranslational protein targeting to membrane
A3LPB2	Antioxidant and copper/iron homeostasis protein	ATX1	80.06	5.36	48.64	3.77	-	Metal ion transport, protection against oxygen radical toxicity and in the delivery of copper to Fet3p
A3LX38	60S ribosomal protein L13	RPL13	22.80	11.08	26.73	3.54	-	Translation, rRNA maturation
A3LNK5	Ribosomal protein L37	PICST_76246	9.86	12.19	30.68	2.03	-	Translation, protein which binds to RNA (RNA binding), metal (Zn) binding
A3LPG9	Predicted protein	PICST_76243	10.81	3.66	88.78	4.72	-	Translational elongation, regulate the activity of the 60S subunit, regulate the pattern of protein expression
A3LYQ3	Frataxin homolog, mitochondrial	FRD1	18.23	4.56	65.45	1.10	-	Iron-sulfur cluster assembly, regulate sensitivity to oxidative stress

*continued on next page*

Table 2 (cont'd)

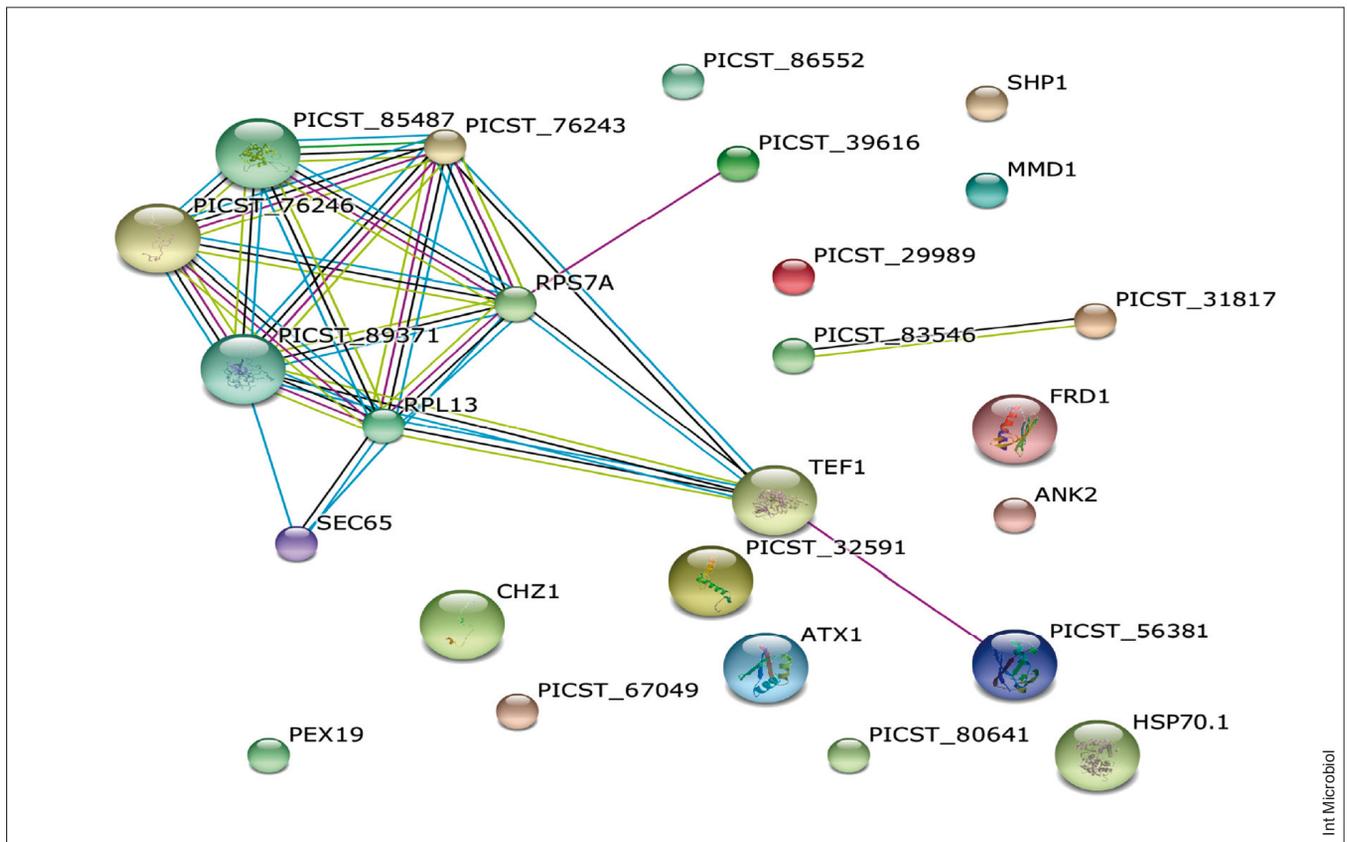
Accession no <sup>a</sup>	Protein name	Gene	MW <sup>b</sup> (kDa)	pI <sup>c</sup>	Coverage (%)	Precursor Mass Error (ppm)	Expression	Function
A3GG84	Hypothetical protein	PICST_86552	7.65	4.68	57.33	4.20	-	Metal detoxification, responsible for high-level copper ion resistance
A3LXX5	Histone H2A.Z-specific chaperone CHZ1	CHZ1	17.24	4.14	62.58	6.06	-	Epigenetic regulation
A3GGK5	Actin binding protein	PICST_80641	65.85	4.52	27.55	4.35	-	Modulator of gene transcription, control the organization of the actin cytoskeleton in a flexible manner
A3LTT0	Respiratory growth induced protein 1	PICST_83546 (RGI1)	23.47	5.39	46.50	5.58	-	Protein of unknown function involved in energy metabolism under respiratory conditions, involve in iron homeostasis
A3LQC6	Elongation factor 1- $\alpha$	TEF1	49.90	9.45	26.63	6.75	-	Translational elongation, regulation of actin cytoskeleton and cell morphology, play central role in regulation of cell signaling
A3GHE8	Predicted protein	PICST_39616	30.14	9.40	57.24	4.02	-	Nucleic acid binding

<sup>a</sup> UniProt accession number.<sup>b</sup> Database molecular weight (MW).<sup>c</sup> Isoelectric point.

strain M12 (Fig. 1A). Transmethylation is an important for preserving yeast cell membrane integrity and for conferring ethanol tolerance [11]. The elimination of this step interrupts ergosterol biosynthesis, resulting in SAM accumulation (Fig. 1A) and a decreased ergosterol level (Fig. 1B). Strain M12, with a lower ergosterol content, was hypersensitive to ethanol (Fig. 1C).

In exploring the molecular mechanism underlying SAM accumulation in strain M12 we found that the mutant lacked detectable Erg6p expression (Figs. 2 and 3). Previous studies of *C. albicans* and *S. cerevisiae* showed that the *ERG6* gene is nonessential for cell viability but critical for the production of ergosterol [16,18,24] and for ethanol tolerance [11,24]. SAM-accumulating strain M12 does not have a mutation in its *ERG6* gene, which suggests that the absence of Erg6p expression is due to defective transcription or translation. In *S. cerevisiae*, *ERG6* expression is regulated at the level of transcription, through the Mot3 protein or by sterol regulatory element binding proteins such as UPC/ECM22 [9, 28].

The physiological characteristics of strain M12 were examined using UPLC-MS<sup>E</sup>. In terms of protein expression, the greatest differences between strain M12 and the wild-type were in proteins involved in protein synthesis and processing and in proteins involved in the stress response (Table 2). In strain M12, four ribosomal proteins involved in translation were underexpressed (ribosomal protein L14b/L23e, ribosomal protein S7A, 60S ribosomal protein L13, ribosomal protein L37) and only one (40S ribosomal protein) was overexpressed compared to the wild-type. The underexpression of several ribosomal proteins may have facilitated isolation of SAM-accumulating strain M12 on medium containing the polyene drug nystatin [13,31]. Ribosome biogenesis is a critical factor in yeast metabolism under ethanol stress [12]. The reduced ethanol tolerance of strain M12 (Fig. 1C) may be related to the underexpression of many ribosomal proteins and to two different non-ribosomal proteins (lethality suppressor and 40kDa farnesylated protein) (Table 2). Proteome analysis showed that respiratory growth reduced protein 1 was not expressed by strain M12. This finding is in agreement with previous studies showing that this protein is not expressed by yeast strains resistant to polyene drugs [6] and by those sensitive to ethanol [12]. The changes in lipid metabolism by strain M12 may be related to the underexpression of the specific chaperone Chz1p (Table 2), which interacts with Htz1p, an expression modulator of many oleate-responsive genes involved in lipid metabolism [30]. The synthesis of lipid components such as sterols or phospholipids is dependent on the transfer of a methyl group from SAM [20]. Thus, our study



**Fig. 4.** Protein–protein interaction map of proteins differentially expressed in *Scheffersomyces stipitis* strain M12 and wild-type *S. stipitis* as determined using the STRING web-tool and the UniProt *S. stipitis* database. The proteins are described in Table 2.

supports an association between ergosterol biosynthesis, ethanol sensitivity, and SAM accumulation.

We also integrated the differentially expressed proteins into a map of protein–protein interactions (Fig. 4). The elucidation of protein–protein interaction networks analysis can shed light on changes in cellular functions, the co-expression and co-regulation of proteins, and phenotypic behavior [10]. The changes in SAM-accumulating *S. stipitis* strain M12 strain (Fig. 4) may therefore be due to the altered expression of proteins involved in protein synthesis and processing and those associated with mitochondria [12,31]. Three proteins (ribosomal protein L16b/L23e, 60S ribosomal protein L13, and ribosomal protein S7A) involved in translation were identified as the main components of the network of proteins involved in protein synthesis and processing in wild-type *S. stipitis* (Fig. 4), based on their large number of interactions [25]. In strain M12, these proteins are replaced by a 40S ribosomal protein and one predicted protein (PICST\_32591), which could explain the changes in the translation of *ERG6* by this strain. However, protein production in yeast cells is

mostly limited by the availability of free ribosomes [22], but in strain M12 strain the predicted protein (PICST\_31817), involved in ribosome biogenesis, was overexpressed. A genomic evaluation of an ethanol-tolerant strain of *S. cerevisiae* also showed changes in protein synthesis and energy metabolism [12].

In summary, the disruption of C-24 methylation in ergosterol biosynthesis in *S. stipitis* strain M12 resulted in the higher accumulation of SAM, a decrease in ergosterol content, and an increased sensitivity to ethanol compared to the wild-type. A proteomic analysis to investigate the changes in the physiological characteristics of *S. stipitis* strain M12 showed that this mutant has an altered proteomic profile compared to the wild-type. Further development of this SAM-accumulating strain could lead to new and innovative therapeutic and commercial applications.

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