J Ind Microbiol Biotechnol (2014) 41:823–835
DOI 10.1007/s10295-014-1422-7

SIMB
Society for Industrial Microbiology and Biotechnology

Metabolic and bioprocess engineering of the yeast *Candida famata* for FAD production

Valentyna Y. Yatsyshyn · Dariya V. Fedorovych · Andriy A. Sibirny

Received: 26 June 2013 / Accepted: 13 February 2014 / Published online: 5 March 2014

Abstract Flavins in the form of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) play an important role in metabolism as cofactors for oxidoreductases and other enzymes. Flavin nucleotides have applications in the food industry and medicine; FAD supplements have been efficiently used for treatment of some inheritable diseases. FAD is produced biotechnologically; however, this compound is much more expensive than riboflavin. Flavinogenic yeast *Candida famata* synthesizes FAD from FMN and ATP in the reaction catalyzed by FAD synthetase, a product of the *FAD1* gene. Expression of *FAD1* from the strong constitutive promoter *TEF1* resulted in 7- to 15-fold increase in FAD synthetase activity, FAD overproduction, and secretion to the culture medium. The effectiveness of FAD production under different growth conditions by one of these recombinant strains, *C. famata* T-FD-FM 27, was evaluated. First, the two-level Plackett–Burman design was performed to screen medium components that significantly influence FAD production. Second, central composite design was adopted to investigate the optimum value of the selected factors for achieving maximum FAD yield. FAD production varied most significantly in response to concentrations of adenine, KH_2PO_4, glycine, and $(NH_4)_2SO_4$. Implementation of these optimization strategies resulted in 65-fold increase in FAD production when compared to the non-optimized control conditions. Recombinant strain that has been cultivated for 40 h under optimized conditions achieved a FAD accumulation of 451 mg/l. So, for the first time yeast strains overproducing FAD were obtained, and the growth media composition for maximum production of this nucleotide was designed.

Keywords FAD · Yeast · *Candida famata* · Metabolic engineering · Optimizing medium components · Central composite design

V. Y. Yatsyshyn · D. V. Fedorovych · A. A. Sibirny (✉)
Department of Molecular Genetics and Biotechnology, Institute of Cell Biology, NAS of Ukraine, Drahomanov Street, 14/16, Lviv 79005, Ukraine
e-mail: sibirny@cellbiol.lviv.ua

A. A. Sibirny
Department of Biotechnology and Microbiology, University of Rzeszow, Zelwerowicza, 4, 35-601 Rzeszow, Poland

Introduction

Flavins play essential biological roles as part of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). These nucleotides bind with proteins to produce the flavoproteins (flavoenzymes). To date, hundreds of flavoproteins are known [20] and new ones are described every year. It is currently estimated that on an average about 1–3 % of the genes in bacterial and eukaryotic genomes encode flavin-binding proteins [4]. Most flavoproteins contain non-covalently bound FAD and, more rarely, FMN.

Only plants, fungi, and most prokaryotes can synthesize riboflavin. However all organisms, including animals, are able to synthesize FMN and FAD from riboflavin [1, 40]. Eukaryotes generally use two different enzymes for FMN and FAD production, whereas most prokaryotes depend on a single bifunctional enzyme, the riboflavin kinase/FAD synthetase [9]. Genes coding for monofunctional eukaryotic FAD synthetases from yeasts [36], mammals, and plants [1, 30] have been cloned and overexpressed. Synthesized FAD can be hydrolyzed to FMN and AMP by nonspecific pyrophosphatases [1].

Flavin nucleotides have applications in pharmacy and the food industry [1, 32]. FAD is used worldwide as an ophthalmic agent and as a component of polyvitamin mixtures (http://www.drugs.com/international/flavin-adenine-dinucleotide.html). Recent reports have shown that FAD treatment could be helpful in some inheritable diseases, e.g., chronic granulomatosis caused by mutations in leukocyte NADPH oxidase genes [13] and Friedreich ataxia caused by the lack of the mitochondrial protein frataxin [10].

FAD is produced biotechnologically, with an annual production of 10 tons [32]; however, this compound is much more expensive than riboflavin. FAD could be isolated from the mycelium of *Eremothecium ashbyii* [21, 38] or produced by biotransformation of exogenous FMN or riboflavin and ATP using bacterial cells [1].

The first published process of FAD production employed a mutant of *Sarcina lutea* defective in adenosine deaminase and a medium supplemented with exogenously added FMN. D-Cycloserine stimulated this process, and the maximal yield of FAD reached 0.7 g/l after 5 days of cultivation [28, 35]. There are some disadvantages of this method: long cultivation period and requirement for expensive chemicals (FMN, D-cycloserine). Enzymatic conversion of FMN and ATP to FAD using FAD synthetase from *Arthrobacter globiformis* is much less productive relative to that of *S. lutea*, as the maximal FAD yield was less than 2 mg/l [33].

More recently, efficient transformation of exogenous FMN and ATP to FAD was achieved using cells of a recombinant strain of *Brevibacterium ammoniagenes* with a 20-fold derepression of bifunctional riboflavin kinase/FAD synthetase [12]. One hundred and sixty milligram per milliliter cells, as an enzyme source, produced 1.2 g FAD/l after 45 h of incubation. Heterologous overexpression of bifunctional riboflavin kinase/FAD synthetase from *Escherichia coli*, *Enterobacter*, and *Pseudomonas* resulted in increased concentrations of FAD (up to 18 g/l) in a medium supplemented with corresponding amounts of FMN and ATP (12 and 24 g/l, respectively) and using 200 mg/ml of cells during 20 h [18]. It has to be pointed out those recombinant bacteria with overexpressed FAD synthetase need high concentrations of expensive additives (FMN and especially ATP) and large amounts of cells. Apparently there is an intrinsic drawback in the development of bacterial producers of flavin nucleotides as FMN is a corepressor of riboflavin-dependent riboswitches (RFN) and prevents accumulation of large amounts of flavin nucleotides by de novo biosynthesis [1].

In this paper, we report the engineering of recombinant strains of the yeast *C. famata* oversynthesizing the FAD de novo. The *FAD1* gene from the flavinogenic yeast *Debaryomyces hansenii* was introduced into the genome of *C. famata* strain T-OP 13–76, which is able to overproduce FMN [39, 41]. Preliminary data on the construction of FAD overproducers were published in short form in our review [40].

The methodology of the next part of this work is based on the Plackett–Burman (PB) design, which provides an efficient way of screening a large number of variables and identifying the most important ones [37]. Response surface methodology (RSM), which includes factorial design and regression analysis, helps in evaluating the effective factors and in building models to study interaction between the variables and also select optimum conditions of variables for a desirable response [16, 26, 37, 39].

We screened the different media components for the best FAD production. We performed a sequential statistical experimental design and formulated an optimal medium for increasing the FAD production by recombinant *C. famata* T-FD-FM 27 strain. In 40 h this strain accumulated 451 mg/l of FAD which was synthesized de novo.

Materials and methods

Yeast strains, media, cultivation conditions

The yeast strains used in this study were *Debaryomyces hansenii* strain CBS 767, *Candida famata* strain VKM Y-9 (it was proposed recently to reclassify this strain as *Candida flareri* by Nguyen et al. [24]), *C. famata* recombinant strain-overproducer of FMN T-OP 13–76 has increased expression of the RF kinase gene (*FMN1*) [39, 41], *C. famata* recombinant strains expressing the *FAD1* gene under the *TEF1* promoter (designated T-FD), and *C. famata* recombinant strains expressing the *FAD1* gene under the *TEF1* promoter and additionally containing the *FMN1* gene under the *TEF1* promoter (designated T-FD-FM).

Yeast cells were cultivated at 28–30 °C in YPD medium (0.5 % yeast extract, 1 % peptone, and 2 % glucose) or modified Burkholder medium (which contained per 1 l: sucrose, 20 g; $(NH_4)_2SO_4$, 3 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 6H_2O$, 0.2 g; biotin, 1 μg. Trace salts at the following final concentrations: 0.2 μM $CuSO_4$, 1.25 μM KI, 4.5 μM $MnSO_4$, 2.0 μM $NaMoO_4$, 0.75 μM H_3BO_3, 17.5 μM $ZnSO_4$).

The *E. coli* strain DH5α [Φ80d*lacZ* ΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17(r_K^-, m_K^+), *supE*44, *relA*1, *deoR*, Δ(*lacZYA-argF*) U 169] was used as a host for selection and propagation of plasmids involved in the study. This strain was grown at 37 °C in LB medium as described previously [29]. Transformed *E. coli* cells were maintained on a medium containing 100 mg/l of ampicillin. When necessary 2 % agar was added to solidify the media.

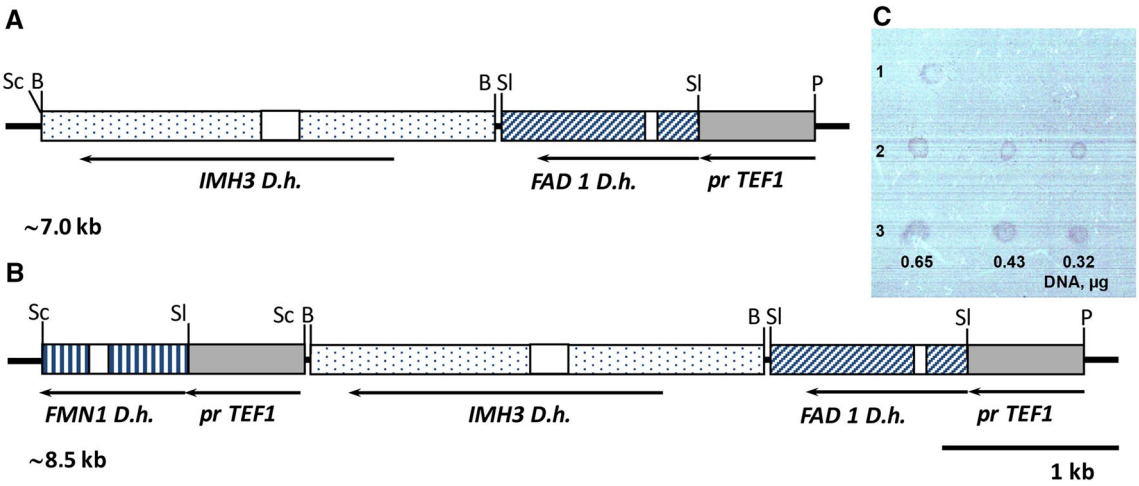

Fig. 1 Linear schemes of constructed plasmids pTFAD1 (**a**), pTFMN1_FAD1 (**b**), Southern blot analysis of yeast genomic DNA for determination of *FAD1 D. hansenii* copies number (**c**). The construct contained a whole sequence of pUC57 (*thin line*); *C. famata* DNA fragment with *IMH3* gene (*dotted box*); *D. hansenii* DNA fragment with *FAD1* gene (*box with slanting hatches*); *D. hansenii* DNA fragment with *FMN1* gene (*box with vertical hatches*); *C. famata* DNA fragment with *TEF1* promoter (*gray box*); introns marked as *open boxes*. Restriction sites: Sc, *Sac*I; B, *Bam*HI; Sl, *Sal*I; P, *Pst*I. Strains used for Southern blot analysis *1 D. hansenii* CBS 767, *2 C. famata* T-FD 3, *3 C. famata* T-FD-FM 27

DNA manipulations

The sequence of *D. hansenii* CBS 767 gene encoding FAD synthetase was obtained from the Genolevures database (DEHA2E13662g; http://www.genolevures.org/elt/DEHA/DEHA2E13662g). The *FAD1* gene encoding FAD synthetase was amplified by PCR from the genomic DNA of *D. hansenii* CBS 767; the *C. famata TEF1* promoter (translation elongation factor 1α) was isolated as described before [14] and amplified by PCR from the genomic DNA of *C. famata* (VKM Y-9).

The plasmid pDhIMH3 bearing *D. hansenii* gene *IMH3* was constructed earlier [5]: the *IMH3* gene was PCR-amplified from genomic DNA of *D. hansenii* CBS767 using a pair of primers Ko90 (5′-CGC GGA TCC GTC GAT GGT CTT GAT CAT TC-3′) and Ko91 (5′-CGC GGATCC GCA TTC TGT TCC AGT TTC TG-3′) and cloned into the *Bam*HI site of the basis plasmids.

The plasmid pTFAD1 (Fig. 1a) was constructed as follows. Designed primers for the *FAD1* gene included sites for *Sal*I (underlined): the forward primer 5′-TATGTCGACATG GAGAACGGAAACGGGG-3′, the reverse primer 5′-CACG TCGACCCTATTAATGTTGGCCATAC-3′. Designed primers for the *TEF1* gene promoter included sites for *Pst*I and *Sal*I, respectively (underlined): the forward primer 5′-TTTCTGCAGTAACGAACA-3′, the reverse primer 5′-GCGGTCGACTTTGCTTAA-3′. Expected fragments of 1,100 bp (the *FAD1* gene) and 658 bp (the *TEF1* promoter) were isolated. The *Pst*I–*Sal*I fragment with *C. famata TEF1* promoter and *Sal*I fragment with *FAD1* ORF of *D. hansenii* were cloned into the plasmid pDhIMH3 [5]. The resulting plasmid was designated pTFAD1 (7 kb).

The plasmid pTFMN1_FAD1 (Fig. 1b) was constructed as follows. The expected fragment of 1,770 bp (*TEF1* promoter and *FAD1* gene) was isolated using the forward primer for the *TEF1* gene promoter and the reverse primer for the *FAD1* gene and the plasmid pTFAD1 as a template. Simultaneously the *Sac*I fragment with *TEF1* promoter and the *FMN1* gene (1,485 bp) was isolated using primers which included sites for *Sac*I (underlined): the forward primer (5′-GGCGAGCTCTAACGAACAGCTCATCAGAT TTAC-3′), the reverse primer (5′-ACAGAGCTCCCTTAAG TAAAAGTACCCCAAAATAGAAC-3′), and the plasmid p19L2_pr*TEF1_FMN1Dh* constructed earlier [15] as a template. The resulting fragment was cloned into the plasmid pDhIMH3 [5] and was designated as pIMH3_TFMN1 (6.75 kb). The *Pst*I–*Sal*I fragment with *C. famata TEF1* promoter and the *FAD1* gene was cloned into the plasmid pIMH3_TFMN1. The resulting plasmid was designated pTFMN1_FAD1 (8.5 kb).

Plasmid and genomic DNA isolation, DNA restriction and ligation, agarose gel electrophoresis, and Southern blot analysis (Fig. 1c) of yeast genomic DNA were carried out as described by Sambrook et al. [29]. *Escherichia coli* transformations were performed by electroporation [29]. *Candida famata* electrotransformations were performed using the method described before [34]: the following electroporation conditions were used: Electro cell manipulator ECM 600 (BTX electroporation system); field strength, 11.5 kV/cm; capacitance, 50 μF; resistance, 129 Ω (R5) resulting in

a pulse length of ±4.5 ms. The transformation frequency for strain T-OP 13 was 75–100 transformants/μg DNA with both plasmids. The presence of recombinant constructs in yeast transformants was confirmed by PCR analysis.

Analytical procedures

Protein quantity was determined by the Lowry method [19]. Riboflavin kinase activity was assayed by the fluorometric method [17]. The assay mixture contained potassium phosphate buffer (pH 8.0) 75 mM, $MgSO_4$ 1 mM, riboflavin 0.1 mM, and ATP 1 mM. FAD pyrophosphatase activity was determined spectrophotometrically [31]. The assay mixture contained potassium phosphate buffer (pH 8.0) 75 mM and FAD 0.025 mM. FAD synthetase was assayed by the fluorometric method [31]. The assay mixture contained potassium phosphate buffer (pH 7.5) 75 mM, $MgCl_2$ 1.5 mM, FMN 0.065 mM, and ATP 2 mM.

Enzyme activities [31] were measured after dialysis of cell-free extracts. Cell-free extracts were prepared by vortexing yeast cells with glass beads in Eppendorf microtubes at 4 °C for 10 min with 0.075 M potassium phosphate buffer pH 7.5. One unit (U) was defined as the amount of the enzyme that catalyzes the conversion of 1 μmole of substrate per minute. The cellular flavin content was measured fluorometrically (Turner Quantech FM 109510-33 fluorometer) after extraction with 5 % trichloracetic acid and neutralization by 2.4 M K_2HPO_4. FAD was identified chromatographically by HPLC (Knauer, Germany) under the following conditions: flow rate, 1 ml/min; eluent, acetonitrile, 0.5 M H_3PO_4 with NaOH pH 3.0 (85:15, v/v); analytical column, Lichrosorb RP18 (250 mm × 4 mm); peak detection at 270/565 nm (excitation and emission). The FAD concentration was measured (as the difference in fluorescence before and after hydrolysis in 0.05 M H_2SO_4) using a Turner Quantech FM 109510-33 fluorometer [8].

Experimental design for optimization

Plackett–Burman design [25]

PB design was introduced as a first optimization step to determine factors that significantly influenced FAD production. The total number of trials to be carried out according to PB is $k + 1$ where k is the number of variables. On the basis of PB design, each variable was examined at two levels: −1 for low level and +1 for high level. In this study, 15 assigned variables were screened in 22 experimental designs (which included six central points). The program Statistica® 6.0 Stat Soft, Inc., was used to analyze the experimental PB design. The factors (Table 1) significant at the 95 % level ($p < 0.05$) were considered to have a significant effect on FAD production and thus were used for further optimization by central composite design (CCD).

Central composite design [22]

On the basis of the results of the PB design, the experiment was further expanded to a CCD. The significant factors identified from PB design, adenine (F14), KH_2PO_4 (F4), glycine (F3) $(NH_4)_2SO_4$ (F2), were chosen as critical factors. Basically this optimization process involves three major steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model, and predicting the response and checking the adequacy of the model. The levels of the variables giving maximum response can then be calculated by using the mathematical model. Each factor in the design was studied at five different levels (−2, −1, 0, +1, +2). A set of 32 experiments was performed. All the variables were taken at a central-coded value considered as zero. The minimum and maximum ranges of variables investigated and the full experimental plan with respect to their values in actual and coded form are listed in Table 2. Upon completion of the experiment, the FAD production was taken as the dependent variable or response (Y). The independent variables are coded for statistical calculation according to the following equation:

$$X_i = \frac{x_i - x_0}{\Delta x_i}, \quad (1)$$

Table 1 Range of different variables studied in the Plackett–Burman design

Variables (g/l)	Variable code	Levels (g/l)		
		Low (−)	Central point (0)	High (+)
Sucrose	F1	20	25	30
$(NH_4)_2SO_4$	F2	2	3	4
Glycine	F3	2	3	4
KH_2PO_4	F4	0.5	1.75	3
$MgSO_4{\cdot}7H_2O$	F5	0.2	0.7	1.2
$CaCl_2{\cdot}6H_2O$	F6	0.2	0.7	1.2
$(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$	F7	0.0012	0.0506	0.1
$ZnSO_4{\cdot}7H_2O$	F8	0.000308	0.050154	0.1
$CuSO_4{\cdot}5H_2O$	F9	0.000039	0.0005195	0.001
$MnSO_4{\cdot}7H_2O$	F10	0.00005	0.000525	0.001
$CoCl_2{\cdot}6H_2O$	F11	0	0.025	0.005
$Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$	F12	0	0.0005	0.001
CH_3COONH_4	F13	0	1	2
Adenine	F14	0	0.05	0.1
Yeast extract	F15	0	0.5	1

Table 2 Variables and their levels for CCD

Variables	Symbols		Levels[a] (g/l)				
	Uncoded	Coded	−2	−1	0	1	2
$(NH_4)_2SO_4$	x_1	X_1	4	5	6	7	8
Glycine	x_2	X_2	4	5	6	7	8
KH_2PO_4	x_3	X_3	3	4	5	6	7
Adenine	x_4	X_4	0.1	0.2	0.3	0.4	0.5

$X_1 = (x_1 - 6)/1$; $X_2 = (x_2 - 6)/1$; $X_3 = (x_3 - 5)/1$; $X_4 = (x_4 - 0.3)/0.1$

[a] Coded level limits are based on preliminary investigations and also to reflect practicality

where X_i is the dimensionless coded value of the independent variable x_i, x_i is the real value of that independent variable, x_0 is the real value of that independent variable x_i at the center point, Δx_i is the step change. The role of each variable, their interactions, and statistical analysis to obtain predicted yields are explained by applying following quadratic equation:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ij} X_i X_j + \Sigma\beta_{ii} X_i^2 \quad (2)$$

where Y is the predicted response, β_0 is an offset term, β_i is the linear effect, β_{ii} is the squared effect, β_{ij} is the interaction effect, X_i and X_j are the levels of the independent variables.

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). Statistical significance of the model equation was determined by Fisher's test value, and the proportion of variance explained by the model was given by the multiple coefficient of determination squared (R^2) value. The software Statistica® 6.0 Stat Soft, Inc. was used in this investigation. All experimental designs were randomized. Experiments were performed in triplicate and mean values were applied.

Results and discussion

Recombinant strains construction

Since the genome of *C. famata* is not sequenced, we overexpressed the *FAD1* gene from flavinogenic strain *D. hansenii*. The sequence of *D. hansenii* CBS 767 putative gene encoding FAD synthetase DEHA2E13662g was obtained from the Genolevures database (http://www.genolevures.org/elt/DEHA/ DEHA2E13662g). *Debaryomyces hansenii* FAD synthetase gene was placed under control of the homologous (*C. famata*) strong constitutive promoter of *TEF1* gene coding for translation elongation factor 1α [14]. To provide augmented FAD synthesis with FMN we have employed a *C. famata* strain overexpressing riboflavin kinase and overproducing FMN [41].

We tested two different constructs for *FAD1* overexpression ("Materials and methods", "DNA manipulations"). The first plasmid, pTFAD1, contained the *FAD1* gene under the *TEF1* promoter (Fig. 1a). To express the *FAD1* gene in *C. famata*, this plasmid was linearized by restriction endonuclease *Pst*I and introduced into *C. famata* T-OP 13–76 by electrotransformation.

The second plasmid pTFMN1_FAD1 contained the *FAD1* gene and an additional copy of the *D. hansenii FMN1* gene under the *TEF1* promoter (Fig. 1b). pTFMN1_FAD1 was linearized by restriction endonuclease *Pst*I and introduced into strain *C. famata* T-OP 13–76. We anticipated that the additional copy of the *FMN1* gene would increase riboflavin kinase activity and thus provide more substrate (FMN) for adenylyltransferase reaction.

Plasmids pTFAD1 and pTFMN1_FAD1 contained the *D. hansenii IMH3* gene conferring the resistance to mycophenolic acid [6, 7]. Transformants were selected on media supplemented with 20 mg/l of mycophenolic acid. The presence of the recombinant *FAD1* gene was confirmed by PCR. Ninety percent of isolated strains appeared to be stable under non-selective conditions. These strains retained resistance to mycophenolic acid and did not lose the recombinant *FAD1* gene. Recombinant strains designated as T-FD were transformed with pTFAD1 and strains designated as T-FD-FM were transformed with pTFMN1_FAD1. All transformants stored at −70 °C have been stable during a 3-year period.

The integration of two copies of the recombinant *FAD1* gene into the genome of the strains with a high level of FAD synthetase activity (see below) was confirmed by Southern hybridization (Fig. 1c).

Biochemical characterization of the recombinant strains

Among flavin-overproducing microorganisms, only *E. ashbyii* [23] and mutant strains of *B. ammoniagenes* and *Micrococcus glutamicus* [40] are capable of producing FAD. Accumulation of FAD in the culture medium was not described for the wild-type yeast strains. At the same time, FAD is the major cellular flavin for both wild-type strains and riboflavin-overproducing strains (cited in [1]).

The intra- and extracellular FAD content (Fig. 2) was measured in recombinant strains of *C. famata* after 48 h of

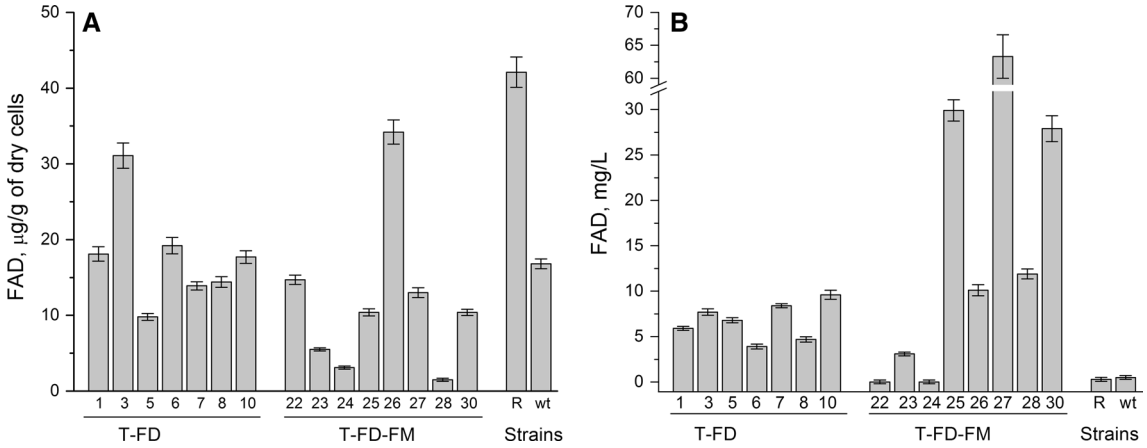

Fig. 2 FAD accumulation in cells (**a**) and in the culture medium (**b**) of *C. famata* recombinant strains: *T-FD* strains contained plasmid pTFAD1 (containing the *FAD1* gene under the *TEF1* promoter); *T-FD-FM* strains contained plasmid pTFMN1_FAD1 (containing the *FAD1* gene under the *TEF1* promoter and the *FMN1* gene under the *TEF1* promoter); *R* recipient strain *C. famata* T-FM 13–76 (containing the *FMN1* gene under the *TEF1* promoter); *wt C. famata* VKM Y-9

growth in liquid Burkholder medium supplemented with 2 g/l of $(NH_4)_2SO_4$ and 2 g/l of glycine as recommended [31]. It was interesting that the wild-type strain and recipient FMN-overproducing strain *C. famata* had different intracellular FAD content (recipient strain T-OP 13–76 accumulates 50 % more FAD than the wild-type strain; Fig. 2a). All analyzed recombinant strains (which contained additional copies of *FAD1* or both *FAD1* and *FMN1* genes) accumulate biomass similar to that of the recipient strain (1.15–1.98 mg/ml after 48 h of growth). The intracellular FAD content (Fig. 2a) in the majority of transformants was close to the wild-type strain intracellular FAD content (2–18 μg/g of dry cells). Only two strains demonstrated more than 30 μg FAD/g of dry cells (as recipient strain *C. famata* T-OP 13–76).

The FAD secretion in the culture medium by the wild-type strain and recipient strain of *C. famata* was extremely low (Fig. 2b) and amounted to 0.4 mg/l. All the strains belonging to group T-FD (which contained additional copies of the *FAD1* gene) accumulated in the culture medium 3.9–9.6 mg/l of FAD (more then tenfold increase in comparison with recipient and wild-type strains). The FAD production in group T-FD-FM (which contained both *FAD1* and *FMN1* genes) was more variable. In two strains FAD in the culture medium was not detected (T-FD-FM 22 and 24). However, most of them accumulated more FAD as compared to T-FD strains and several of them (T-FD-FM 25, 27, 30) accumulated much more FAD (about 30–60 mg/l after 48 h of growth) in the culture medium.

The total extracellular flavins content (riboflavin + FMN + FAD) in all the recombinant strains was not different from that in the recipient strain (400 mg/l), so only FAD percentage varied (1–2.5 % in strains belonging to group T-FD and up to 18 % in strains with the highest FAD amount; data not shown). The recombinant strains of the T-FD series accumulated FMN in the medium similar to parental strain T-OP 13–76 (87–92 mg/l for recombinant strains and 96 mg/l for parental strain), whereas recombinant strains of the T-FD-FM series accumulated, as expected, more FMN (101–154 mg/l).

The FAD synthetase activity (Table 3) of all recombinant strains was 6–17 times higher than the activity of the recipient strain and the wild-type strain of *C. famata*. Interestingly, strains T-FD-FM 22 and 24, which contained additional copies of *FAD1* and *FMN1* genes and did not accumulate FAD in the culture medium, show elevated FAD synthetase activity relative to the parental strain.

The activity of FAD pyrophosphatase (an enzyme which hydrolyzes FAD) was similar in obtained transformants, recipient strain, and wild-type strain (0.3–0.7 mU/mg of protein). As expected, the riboflavin kinase activity of recombinant strains belonging to the group T-FD (which contained additional copies of the *FAD1* gene) was similar to those of the recipient strain. Riboflavin kinase activity of recombinant strains belonging to the group T-FD-FM (which contained additional copies of *FAD1* and *FMN1* genes) was 3- to 4-fold higher in comparison with the recipient strain *C. famata* T-OP 13–76.

Thus, overexpression of the *FAD1* gene (encoding the FAD synthetase) in the FMN-overproducing *C. famata* strain resulted in 7- to 15-fold increase of FAD synthetase activity. We suggest that synthesis of FMN is limited to activities of riboflavin kinase [41] and that of FAD by both riboflavin kinase and FAD synthetase (this work).

We decided to achieve increased accumulation of FAD in the culture medium by optimization of the cultivation conditions. For this, the strain *C. famata* T-FD-FM 27 (which contained additional copies of *FAD1* and *FMN1*

Table 3 Riboflavin kinase, FAD synthetase, and FAD pyrophosphatase activities in *C. famata* strains after 48 h of growth

Strain	Enzyme activity, mU/mg of protein		
	Riboflavin kinase	FAD synthetase	FAD pyrophosphatase
T-FD 1	1.98 ± 0.100	0.35 ± 0.015	0.53 ± 0.025
T-FD 3	2.16 ± 0.112	0.40 ± 0.022	0.57 ± 0.022
T-FD 5	2.68 ± 0.109	0.76 ± 0.041	0.45 ± 0.023
T-FD 6	2.79 ± 0.129	0.56 ± 0.029	0.38 ± 0.020
T-FD 7	3.30 ± 0.169	0.36 ± 0.018	0.49 ± 0.027
T-FD 8	2.77 ± 0.133	0.66 ± 0.029	0.57 ± 0.023
T-FD 10	2.93 ± 0.149	0.42 ± 0.019	–
T-FD-FM 22	8.83 ± 0.234	0.58 ± 0.001	0.47 ± 0.021
T-FD-FM 23	7.73 ± 0.213	0.56 ± 0.009	0.41 ± 0.021
T-FD-FM 24	6.88 ± 0.511	0.42 ± 0.014	0.46 ± 0.021
T-FD-FM 25	8.22 ± 0.655	0.40 ± 0.013	0.36 ± 0.015
T-FD-FM 26	7.36 ± 0.322	0.28 ± 0.011	0.54 ± 0.027
T-FD-FM 27	7.73 ± 0.111	0.35 ± 0.008	0.47 ± 0.022
T-FD-FM 28	8.22 ± 0.221	0.57 ± 0.022	0.73 ± 0.031
T-FD-FM 30	6.75 ± 0.557	0.35 ± 0.005	0.33 ± 0.014
T-FM 13–76 (R)	2.25 ± 0.115	0.04 ± 0.004	0.51 ± 0.024
VKM Y-9 (wt)	0.15 ± 0.075	0.05 ± 0.001	0.42 ± 0.019

genes), which produced 63.3 mg/l FAD after 48 h of growth (Fig. 2b), was used.

Screening of significant factors affecting FAD production

It is known that medium composition has a significant influence on FAD production in *C. ammoniagenes*. For example, divalent cations enhanced the conversion of riboflavin to FAD twice [12]. FAD synthetase activity and FAD content in cells are known to be elevated under increasing demand for FAD (cultivation on methanol, glycine, or *n*-alkanes as carbon sources) [2, 31].

A series of experiments were first carried out to study the effects of various medium components on FAD production by the *C. famata* T-FD-FM 27 strain (which contains additional copies of *FAD1* and *FMN1* genes). It was found earlier [41] that high density cultivation of the recombinant cells increased the FMN production in FMN-overproducing strains *C. famata*. So, for optimization experiments high density cultivation (3 mg/ml of yeast cells) has been used. It was shown [12, 18] that the highest level of FAD production in *C. ammoniagenes* was carried out using 100–200 mg/ml cells as an enzyme source. However, in our growth experiments further increasing of initial cell density was not effective for increasing FAD production (data not shown).

Generally, ammonium salt is a preferred inorganic nitrogen source for microorganism growth in most fermentation processes. Figure 3 presents the data on FAD

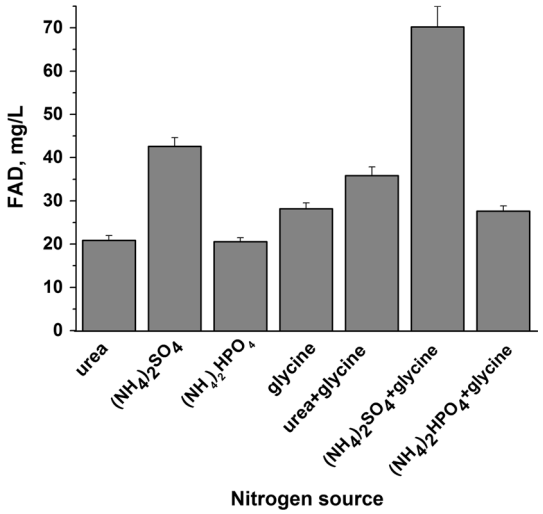

Fig. 3 Effect of different nitrogen sources on FAD production by *C. famata* T-FD-FM 27

production in the media containing different nitrogen sources, such as $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, urea, and the combination of these compounds with glycine. The initial medium contained 20 g/l sucrose, 0.5 g/l KH_2PO_4, 0.2 g/l $MgSO_4 \cdot 7H_2O$, 0.2 g/l $CaCl_2$, trace elements, biotin (see "Materials and methods"), and the defined nitrogen source. The FAD production was largely affected by the nitrogen source with a fourfold difference between certain nitrogen sources. $(NH_4)_2SO_4$ in combination with glycine was the most appropriate for FAD production. It is interesting that the positive effect of glycine was reduced when $(NH_4)_2HPO_4$ was used as the nitrogen source. As a result the medium supplemented with $(NH_4)_2SO_4$ and glycine led to enhanced FAD production of up to 70 mg/l in 15–24 h, so we used 24 h as an appropriate time for ending the shake-flask cultures. Thus $(NH_4)_2SO_4$ and glycine were used as the nitrogen sources for further optimization steps.

PB design was then used as a screening method for detection of variables which have a significant effect on FAD production. The experimental design and corresponding FAD production (mg/l) are shown in Table 4. The effect of each variable on FAD biosynthesis was determined as the difference between the average of measurements made at the high level and the low level of the factor. The medium components screened contained sucrose $(NH_4)_2SO_4$, glycine, KH_2PO_4, $MgSO_4$, $CaCl_2$ $(NH_4)_6Mo_7O_{24}$, $ZnSO_4$, $CuSO_4$, $MnSO_4$, $CoCl_2$, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, ammonium acetate, adenine, and yeast extract. The limits of each variable were chosen to encompass the ranges reported in the literature and to reflect what was done in practice after preliminary investigations.

Figure 4 shows the effects of the variables on the response and significance levels. Confidence levels were

Table 4 Plackett–Burman design matrix for evaluating factors influencing FAD production by *C. famata* T-FD-FM 27

Run	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14	F15	FAD (mg/l)
1	−	−	−	−	+	+	+	+	+	+	−	−	−	−	+	0.05
2	+	−	−	−	−	−	−	+	+	+	+	+	+	−	−	14.01
3	−	+	−	−	−	+	+	−	−	+	+	+	−	+	−	164.16
4	+	+	−	−	+	−	−	−	−	+	−	−	+	+	+	192.19
5	−	−	+	−	+	−	+	−	+	−	+	−	+	+	−	240.24
6	+	−	+	−	−	+	−	−	+	−	−	+	−	+	+	108.10
7	−	+	+	−	−	−	+	+	−	−	−	+	+	−	+	171.17
8	+	+	+	−	+	+	−	+	−	−	+	−	−	−	−	85.08
9	−	−	−	+	+	+	−	+	−	−	−	+	+	+	−	236.23
10	+	−	−	+	−	−	+	+	−	−	+	−	−	+	+	258.25
11	−	+	−	+	−	+	−	−	+	−	+	−	+	−	+	120.12
12	+	+	−	+	+	−	+	−	+	−	−	+	−	−	−	111.11
13	−	−	+	+	+	−	−	−	−	+	+	+	−	−	+	236.23
14	+	−	+	+	−	+	+	−	−	+	−	−	+	−	−	173.17
15	−	+	+	+	−	−	−	+	+	+	−	−	−	+	−	482.48
16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	557.55
17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	286.28
18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	271.17
19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	205.20
20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	240.24
21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	268.26
22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	270.57

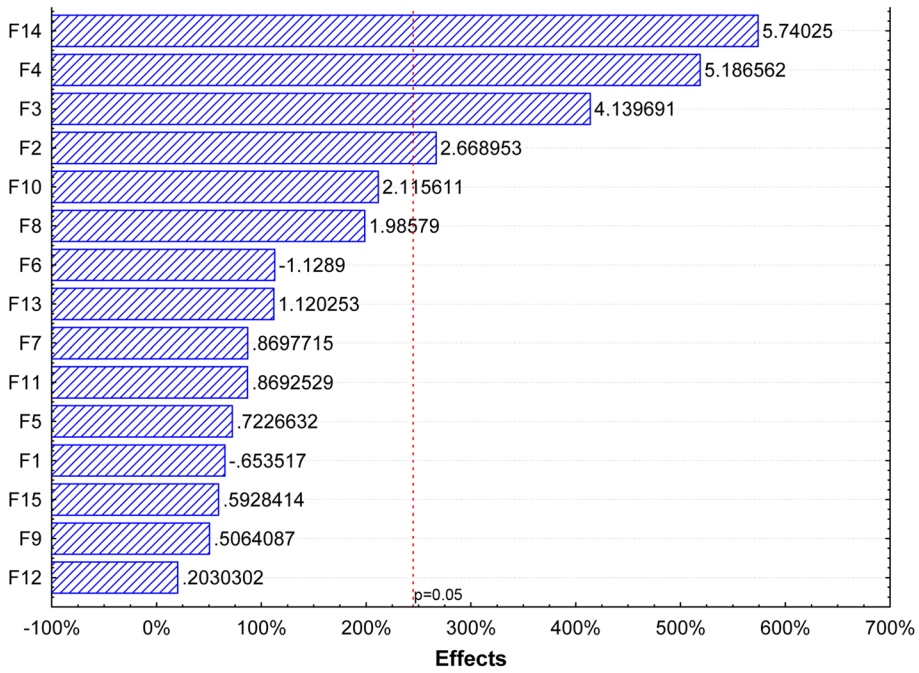

Fig. 4 Pareto charts of standardized effects for FAD production

accepted only when these were above 95 % ($p < 0.05$). On the basis of the statistical analysis, the factors having the greatest impact on the production of FAD by *C. famata* T-FD-FM 27 were identified as F14 (adenine), F4 (KH_2PO_4), F3 (glycine), and F2 [$(NH_4)_2SO_4$; Fig. 4].

These components could be ranked as adenine > KH_2PO_4 > glycine > $(NH_4)_2SO_4$. The effects of these variables were all positive because the influence of the variables upon FAD production was greater in a high concentration. The positive effects of F10 ($MnSO_4$), F8

($ZnSO_4$), F13 (ammonium acetate), F7 [$(NH_4)_6Mo_7O_{24}$], F11 ($CoCl_2$), F5 ($MgSO_4$), F15 (yeast extract), F9 ($CuSO_4$), and F12 [$Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$] were insignificant. Only variables F6 ($CaCl_2$) and F1 (sucrose) were set at their high levels owing to their negative effects. The negative influence of those components is insignificant.

Central composite design

Designing an appropriate fermentation medium is of critical importance in optimizing the product yield; however, the conventional experimental approaches for the medium optimization are time consuming and require a large number of experiments to study the influence of each on the product biosynthesis. On the contrary, statistical optimization of media components can take into account the interactions of variables in generating process responses. Response surface methodology (RSM), which is the most accepted statistical technique for bioprocess optimization, can be used to examine the relationship between a set of controllable experimental factors and observed results [3, 27].

The combined effect of the nitrogen sources [$(NH_4)_2SO_4$ and glycine], phosphate source (KH_2PO_4), and adenine was investigated using CCD, which can help to identify the interaction between the variables. On the basis of the results of the initial PB design, the concentration of F14 (adenine), F4 (KH_2PO_4), F3 (glycine), and F2 [$(NH_4)_2SO_4$] were further optimized using a CCD. The experimental design and results of the CCD are presented in Table 5. In addition, the predicted responses obtained from the statistical analysis are also shown there. In order to determine the optimal value, an analysis of variance was carried out. By applying multiple regression analysis on the experimental data, a second-order polynomial equation was obtained to describe the correlation between the significant variables and the FAD yield in terms of decoded values (Eq. 3).

$$\begin{aligned} Y = {} & 326.287 - 5.709X_1 + 11.383X_2 + 2.049X_3 \\ & + 19.399X_4 - 47.401X_1^2 - 29.638X_2^2 - 22.501X_3^2 \\ & - 7.488X_4^2 + 1.176X_1X_2 - 15.461X_1X_3 - 13.199X_1X_4 \\ & - 19.586X_2X_3 - 1.324X_2X_4 - 15.961X_3X_4, \end{aligned} \tag{3}$$

where Y is the predicted response of FAD production (mg/l), and X_1, X_2, X_3, and X_4 are the coded values of $(NH_4)_2SO_4$, glycine, KH_2PO_4, and adenine, respectively.

The ANOVA was conducted to test the significance of the fit of the second-order polynomial equation for the FAD production as shown in Table 6. The goodness-of-fit of the model was checked by determining the coefficient of determination (R^2) and adjusted R^2. When R^2 is large, the regression has accounted for a large proportion of the total variability in the observed value of Y which favors the regression equation model [11, 27]. The coefficient of determination R^2 was 0.97985, indicating that the response model could explain 97.99 % of the total variations and implied that the correlation between the experimental and predicted values was good. The value of adjusted determination coefficient $\left(R^2_{\text{Adj}} = 0.96326\right)$ was also high enough to indicate the significance of the model. The model F value of 59.05 also implied that the model was significant. The Fisher's F test with a low p value (<0.0001) also indicated that the model was significant. Table 6 displays the analyses of variance for the full second-order polynomial quadratic model. These results reinforced that the response equation provided a suitable model for the CCD experiment.

Three-dimensional response surface plots were employed to demonstrate the interaction among the medium components and to determine the optimum levels of components supplemented into the basal medium, which have significant effects on product biosynthesis. The response surface plots obtained from Eq. (3) are shown in Fig. 5, which illustrates the relationships between response and the experimental data as a function of two variables with the other nutrients being at their constant levels. The shapes of contour plots indicate the nature and extent of the interactions. The prominent interactions of significant components are shown by the elliptical nature of the contour plot in Fig. 5. The response surface of FAD production showed a clear peak, indicating that the optimum conditions were well within the design boundary. FAD synthesis increased with increasing $(NH_4)_2SO_4$, glycine, KH_2PO_4, and adenine concentrations to optimum concentrations and then decreased. The optimal values of variables are obtained when moving along the major and minor axes of the ellipse and the response at the center point yields maximum FAD production.

The optimal values of X_1, X_2, X_3, and X_4 in the coded units were found to be −0.246, 0.443, −0.941, and 2.475, respectively. Correspondingly, we can obtain the maximum points of the models, which were 5.75 g/l $(NH_4)_2SO_4$, 6.44 g/l glycine, 4.06 g/l KH_2PO_4, and 0.55 g/l adenine, respectively. The maximum predicted value of FAD production obtained was 352.55 mg/l.

Validation of the model

To confirm these data, FAD production by *C. famata* was carried out under the optimum conditions. According to the results of the statistical design, the optimized medium was prepared as follows (gram per liter): sucrose, 20; $(NH_4)_2SO_4$, 5.75; glycine, 6.44; KH_2PO_4, 4.06; $MgSO_4{\cdot}7H_2O$, 0.2; $CaCl_2$, 0.2; adenine, 0.55; biotin, 1 μg; trace salts at the following final concentrations: 0.2 μM

Table 5 CCD matrix of variables with experimental and predicted values of FAD production (the levels for each variable are shown in Table 2)

Run no.	Variables				FAD production (mg/l)		
	X_1	X_2	X_3	X_4	Experimental value	Predicted value	Residual value
1	−1	−1	−1	−1	127.020	127.783	−0.763
2	−1	−1	−1	1	228.200	227.548	0.652
3	−1	−1	1	−1	233.200	233.898	−0.698
4	−1	−1	1	1	270.300	269.819	0.481
5	−1	1	−1	−1	190.200	190.015	0.185
6	−1	1	−1	1	284.300	284.486	−0.186
7	−1	1	1	−1	217.200	217.786	−0.586
8	−1	1	1	1	248.200	248.412	−0.212
9	1	−1	−1	−1	171.200	171.332	−0.132
10	1	−1	−1	1	218.300	218.303	−0.003
11	1	−1	1	−1	215.200	215.602	−0.403
12	1	−1	1	1	198.200	198.728	−0.528
13	1	1	−1	−1	237.200	238.269	−1.069
14	1	1	−1	1	280.300	279.945	0.355
15	1	1	1	−1	203.200	204.195	−0.995
16	1	1	1	1	182.200	182.026	0.174
17	−2	0	0	0	148.200	148.102	0.097
18	2	0	0	0	126.100	125.266	0.834
19	0	−2	0	0	185.200	184.969	0.231
20	0	2	0	0	231.200	230.499	0.701
21	0	0	−2	0	232.200	232.186	0.014
22	0	0	2	0	241.300	240.383	0.917
23	0	0	0	−2	259.300	257.536	1.764
24	0	0	0	2	334.300	335.132	−0.832
25	0	0	0	0	317.300	326.287	−8.987
26	0	0	0	0	322.000	326.287	−4.287
27	0	0	0	0	316.300	326.287	−9.987
28	0	0	0	0	317.400	326.287	−8.887
29	0	0	0	0	371.300	326.287	45.013
30	0	0	0	0	318.400	326.287	−7.887
31	0	0	0	0	314.300	326.287	−11.987
32	0	0	0	0	333.300	326.287	7.013

$CuSO_4$, 4.5 μM $MnSO_4$, 2.0 μM $NaMoO_4$, 0.75 μM H_3BO_3, 17.5 μM $ZnSO_4$.

Validation experiments were carried out in 100-ml shake flasks containing 10 ml medium under conditions predicted by the model. The results of a random set of three experiments clearly showed that experimental values were very close to the predicted values, and the model was successfully validated. The predicted response for FAD production was 352.55 mg/l, whereas the actual (experimental) response was 387 mg/l (Fig. 6), which was 5.47-fold higher than the medium supplemented with $(NH_4)_2SO_4$ and glycine (as nitrogen sources; 70.8 mg/l) and 65.8-fold higher than the unoptimized medium (5.88 mg/l). The good agreement between the predicted and the experimental results verified the validity of the model, and the improvement of FAD production also indicated that RSM was a powerful tool for determining the exact optimal values of the individual factors and the maximum response value.

FAD production in bioreactor

The validation of the statistically optimized medium for the production of FAD by *C. famata* T-FD-FM 27 was also verified by carrying out batch fermentation in a 1.2-l fermenter. The composition of fermentation medium for the fermenter was the same as the optimized medium in shake flasks. The medium for FAD production in the bioreactor was inoculated with 1.9–2.0 mg of cells (dry weight) per milliliter.

Table 6 Analysis of variance (ANOVA) for the model regression representing FAD production

Factor	Sum of squares (SS)	Degrees of freedom (*df*)	Mean square (MS)	*F* value	*P* value	Significance
Model	124,994.9	14	8,928.21	59.0597	<0.0001	Significant
X_1	782.3	1	782.27	2.1398	0.1869	
X_2	3,109.5	1	3,109.47	8.5054	0.0225	Significant
X_3	100.8	1	100.78	0.2757	0.6158	
X_4	9,031.9	1	9,031.86	24.7051	0.0016	Significant
X_1X_2	22.1	1	22.14	0.0606	0.8127	
X_1X_3	3,824.8	1	3,824.80	10.4621	0.0144	Significant
X_1X_4	2,787.3	1	2,787.31	7.6242	0.0280	Significant
X_2X_3	6,137.9	1	6,137.94	16.7893	0.0046	Significant
X_2X_4	28.0	1	28.04	0.0767	0.7898	
X_3X_4	4,076.2	1	4,076.18	11.1497	0.0124	Significant
X_1^2	66,368.2	1	66,368.17	181.5387	<0.0001	Significant
X_2^2	25,947.5	1	25,947.49	70.9749	0.0001	Significant
X_3^2	14,955.0	1	14,954.95	40.9067	0.0004	Significant
X_4^2	1,656.4	1	1,656.37	4.5307	0.0708	

Confidence intervals above 95 % ($p < 0.05$) are considered significant

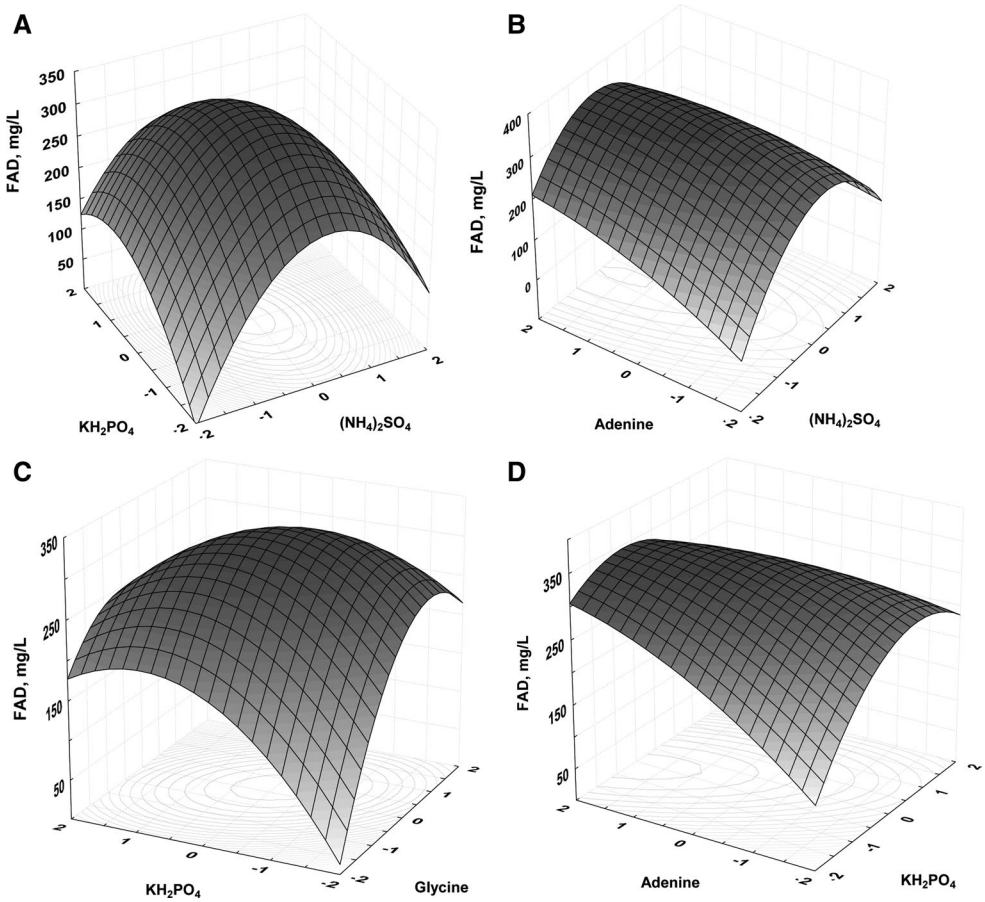

Fig. 5 Response surface plots of FAD production as a function of $(NH_4)_2SO_4$ and KH_2PO_4 (**a**), $(NH_4)_2SO_4$ and adenine (**b**), KH_2PO_4 and glycine (**c**), and KH_2PO_4 and adenine (**d**) concentrations in coded levels (Table 2)

The maximal FAD yield of 451.5 mg/l obtained after 40 h batch cultivation was higher than that observed in shake-flask experiments. The decrease in FAD accumulation in the culture medium after 40 h of cultivation could be explained by FAD hydrolysis, which was confirmed by the increase in riboflavin production at that period of time (Fig. 6).

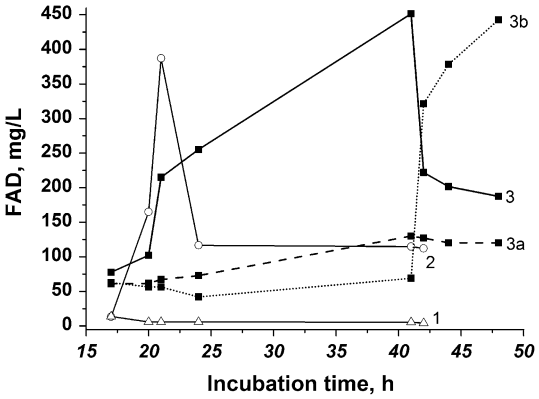

Fig. 6 Flavins production by *C. famata* T-FD-FM 27 batch culture in shake flasks (unoptimized medium: *line 1* FAD production; optimized medium: *line 2* FAD production) and fermenter (*line 3* FAD production; *line 3a* FMN production; *line 3b* riboflavin production). The results of representative experiments were given

Conclusion

The work was devoted to the design of yeast overproducers of FAD using metabolic engineering. For the first time yeast strains overproducing FAD de novo were isolated, and the growth media composition for maximum production of FAD was proposed. The recombinant strains of *C. famata* showed 7- to 15-fold increase in FAD synthetase activity (as compared to the wild-type strain) and some of them accumulated FAD in the culture medium. We also showed that RSM medium optimization increased FAD production from 5.88 mg/l in unoptimized medium to 387.0 mg/l, giving 65.8-fold increase in FAD production. The high correlation between the predicted and observed values indicated the validity of the model. The maximum FAD yield of 451.5 mg/l was obtained after 40 h batch fermenter cultivation in the mineral medium.

In contrast, known bacterial producers of FAD require addition of the expensive FAD precursors (FMN and ATP) and long growth times (in the case of *S. lutea*) or huge cell density (in the case of recombinant *C. ammoniagenes* enzymatic conversion) for FAD production.

We are bound to isolate strains with further increased FAD yield and productivity by overexpressing the *FMN1* and *FAD1* genes in the best riboflavin-overproducing *C. famata* strain, which was isolated recently (K. Dmytruk, A. Sibirny, in preparation). We also suggest that identification and knock out of the pyrophosphatase genes, involved in FAD hydrolysis, would have a positive impact on FAD accumulation by recombinant strains of *C. famata*. These and other approaches (including multicopy integration of the *FAD1* gene) can open the possibility to develop new feasible technology for industrial production of FAD.

References

1. Abbas C, Sibirny A (2011) Genetic control of biosynthesis and transport of riboflavin and flavin nucleotides and construction of robust biotechnological producers. Microbiol Mol Biol Rev 75:321–360. doi:10.1128/MMBR.00030-10
2. Brooke AG, Dijkhuizen L, Harder W (1986) Regulation of flavin biosynthesis in the methylotrophic yeast *Hansenula polymorpha*. Arch Microbiol 145(2):162–170
3. Cao G, Ren N, Wang A, Guo W, Yao J, Feng Y, Zhao Q (2010) Statistical optimization of culture condition for enhanced hydrogen production by *Thermoanaerobacterium thermosaccharolyticum* W16. Bioresour Technol 101(6):2053–2058. doi:10.1016/j.biortech.2009.11.031
4. De Colibus L, Mattevi A (2006) New frontiers in structural flavoenzymology. Curr Opin Struct Biol 16(6):722–728. doi:10.1016/j.sbi.2006.10.003
5. Dmytruk KV, Voronovsky AY, Sibirny AA (2006) Insertion mutagenesis of the yeast *Candida famata* (*Debaryomyces hansenii*) by random integration of linear DNA fragments. Curr Genet 50(3):183–191. doi:10.1007/s00294-006-0083-0
6. Dmytruk KV, Yatsyshyn VY, Sybirna NO, Fedorovych DV, Sibirny AA (2011) Metabolic engineering and classic selection of the yeast *Candida famata* (*Candida flareri*) for construction of strains with enhanced riboflavin production. Metab Eng 13(1):82–88. doi:10.1016/j.ymben.2010.10.005
7. Exinger F, Lacroute F (1992) 6-Azauracil inhibition of GTP biosynthesis in *Saccharomyces cerevisiae*. Curr Genet 22(1):9–11. doi:10.1007/BF00351735
8. Faeder EJ, Siegel LM (1973) A rapid micromethod for determination of FMN and FAD in mixtures. Anal Biochem 53(1):332–336. doi:10.1016/0003-2697(73)90442-9
9. Frago S, Velázquez-Campoy A, Medina M (2009) The puzzle of ligand binding to *Corynebacterium ammoniagenes* FAD synthetase. J Biol Chem 284(11):6610–6619. doi:10.1074/jbc.M808142200
10. Gonzalez-Cabo P, Ros S, Palau F (2010) Flavin adenine dinucleotide rescues the phenotype of frataxin deficiency. PLoS One 5(1):e8872. doi:10.1371/journal.pone.0008872
11. Haaland PD (1990) Experimental design in biotechnology. Elsevier, New York
12. Hagihara T, Fujio T, Aisaka K (1995) Cloning of FAD synthetase gene from *Corynebacterium ammoniagenes* and its application to FAD and FMN production. Appl Microbiol Biotechnol 42(5):724–729
13. Huang YF, Liu SY, Yen CL, Yang PW, Shieh CC (2009) Thapsigargin and flavin adenine dinucleotide ex vivo treatment rescues trafficking-defective gp91phox in chronic granulomatous disease leukocytes. Free Radic Biol Med 47(7):932–940. doi:10.1016/j.freeradbiomed.2009.06.037
14. Ishchuk OP, Dmytruk KV, Rohulya OV, Voronovsky AY, Abbas CA, Sibirny AA (2008) Development of a promoter assay system for the flavinogenic yeast *Candida famata* based on the *Kluyveromyces lactis* β-galactosidase *LAC4* reporter gene. Enzym Microb Technol 42(3):208–215. doi:10.1016/j.enzmictec.2007.09.008
15. Ishchuk OP, IatsyshynViu, Dmytruk KV, Voronovs'kyĭ Aia, Fedorovych DV, Sybirnyĭ AA (2006) Construction of the flavinogenic yeast *Candida famata* strains with high riboflavin kinase activity using gene engineering. Ukr Biokhim Zh 78(5):63–69 (Article in Ukrainian)
16. Kalingan AE, Liao C-M (2002) Influence of type and concentration of flavinogenic factors on production of riboflavin by *Eremothecium ashbyii* NRRL 1363. Bioresour Technol 82(3):219–224. doi:10.1016/S0960-8524(01)00194-8

17. Kashchenko VE, Shavlovskiĭ GM (1976) Purification and properties of the riboflavin kinase of the yeast *Pichia guilliermondii*. Biokhimiia 41(2):376–383 Article in Russian
18. Kitatsuji K, Ishino S, Teshiba S, Arimoto M (1996) Method of producing flavine nucleotides. US Patent 5,514,574
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193(1):265–275
20. Massey V (2000) The chemical and biological versatility of riboflavin. Biochem Soc Trans 28:283–296. doi:10.1074/jbc.M103114200
21. Masuda T (1955) Application of chromatography. XXVIII. On the formation of FAD in the culture of *Eremothecium ashbyii*. Pharm Bull 3:434–440
22. Montgomery DC (1997) Design and analysis of experiments. Wiley, New York
23. Nagatsu T, Nagatsu-Ishibashi I, Yagi K (1963) Biosynthesis of C14-labelled flavin adenine dinucleotide by *Eremothecium ashbyii*. J Biochem 54:152–155 (Tokyo)
24. Nguyen H-V, Gaillardin C, Neuveglise C (2009) Differentiation of *Debaryomyces hansenii* and *Candida famata* by rRNA gene intergenic spacer fingerprinting and reassessment of phylogenetic relationships among *D. hansenii*, *C. famata*, *D. fabryi*, *C. flareri* (= *D. subglobosus*) and *D. prosopidis*: description of *D. vietnamensis* sp. nov. Closely related to *D. nepalensis*. FEMS Yeast Res 9(4):641–662. doi:10.1111/j.1567-1364.2009.00510.x
25. Plackett RL, Burman JP (1946) The design of optimum multifactorial experiments. Biometrika 33(4):305–325
26. Pujari V, Chandra TS (2000) Statistical optimization of medium components for enhanced riboflavin production by a UV-mutant of *Eremothecium ashbyii*. Process Biochem 36(1–2):31–37. doi:10.1016/S0032-9592(00)00173-4
27. Rai SK, Mukherjee AK (2010) Statistical optimization of production, purification and industrial application of a laundry detergent and organic solvent-stable subtilisin-like serine protease (Alzwiprase) from *Bacillus subtilis* DM-04. Biochem Eng J 48:173–180. doi:10.1016/j.bej.2009.09.007
28. Sakai T, Watanabe T, Chibata I (1973) Selection of microorganism producing flavin-adenine dinucleotide from FMN and adenine (AMP) and production of flavin-adenine dinucleotide by *Sarcina lutea*. Agric Biol Chem 37:849–856
29. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor
30. Sandoval FJ, Zhang Y, Roje S (2008) Flavin nucleotide metabolism in plants: monofunctional enzymes synthesize FAD in plastids. J Biol Chem 283(45):30890–30900. doi:10.1074/jbc.M803416200
31. Shavlovskii GM, Fedorovich DV (1977) The activity of enzymes involved in synthesis and hydrolysis of flavin adenine dinucleotide in *Pichia guilliermondii*: studies at different levels of flavinogenesis. Mikrobiologiia 46:904–911 (In Russian)
32. Shimizu S (2001) Vitamins and related compounds: microbial production. In: Rehm H (ed) Biotechnology, vol 10., Wiley-VCHWeinheim, Germany, pp 322–326
33. Shimizu S, Yamane K, Tani Y, Yamada H (1983) Enzymatic synthesis of flavin adenine dinucleotide. Appl Biochem Biotechnol 8:237–247
34. Voronovsky AA, Abbas CA, Fayura LR, Kshanovska BV, Dmytruk KV, Sybirna KA, Sibirny AA (2002) Development of a transformation system for the flavinogenic yeast *Candida famata*. FEMS Yeast Res 2:381–388. doi:10.1016/S1567-1356(02)00112-5
35. Watanabe T, Uchida T, Kato J, Chibata I (1974) Production of flavine-adenine dinucleotide from riboflavine by a mutant of *Sarcina lutea*. Arch Microbiol 27:531–536
36. Wu M, Repetto B, Glerum DM, Tzagoloff A (1995) Cloning and characterization of *FAD1*, the structural gene for flavin adenine dinucleotide synthetase of *Saccharomyces cerevisiae*. Mol Cell Biol 15:264–271
37. Wu QL, Chen T, Gan Y, Chen X, Zhao XM (2007) Optimization of riboflavin production by recombinant *Bacillus subtilis* RH44 using statistical designs. Appl Microbiol Biotechnol 76:783–794. doi:10.1007/s00253-007-1049-y
38. Yagi K, Matsuoka Y, Kuyama S, Tada M (1956) Preparation of flavin adenine dinucleotide from *Eremothecium ashbyii*. J Biochem 43:93–100
39. Yatsyshyn VY, Fedorovych DV, Sibirny AA (2010) Medium optimization for production of flavin mononucleotide by the recombinant strain of the yeast *Candida famata* using statistical designs. Biochem Eng J 49(1):52–60. doi:10.1016/j.bej.2009.11.010
40. Yatsyshyn VY, Fedorovych DV, Sibirny AA (2009) The microbial synthesis of flavin nucleotides: a review. Appl Biochem Microbiol 45(2):115–124. doi:10.1134/S000368380902001X
41. Yatsyshyn VY, Ishchuk OP, Voronovsky AY, Fedorovych DV, Sibirny AA (2009) Production of flavin mononucleotide by metabolically engineered yeast *Candida famata*. Metab Eng 11:163–167. doi:10.1016/j.ymben.2009.01.004