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Regular paper

The sequence diversity and expression among genes of the folic acid biosynthesis pathway in industrial *Saccharomyces* strains*

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Folic acid is an important vitamin in human nutrition and its deficiency in pregnant women's diets results in neural tube defects and other neurological damage to the fetus. Additionally, DNA synthesis, cell division and intestinal absorption are inhibited in case of adults. Since this discovery, governments and health organizations worldwide have made recommendations concerning folic acid supplementation of food for women planning to become pregnant. In many countries this has led to the introduction of fortifications, where synthetic folic acid is added to flour. It is known that *Saccharomyces* strains (brewing and bakers' yeast) are one of the main producers of folic acid and they can be used as a natural source of this vitamin. Proper selection of the most efficient strains may enhance the folate content in bread, fermented vegetables, dairy products and beer by 100% and may be used in the food industry. The objective of this study was to select the optimal producing yeast strain by determining the differences in nucleotide sequences in the *FOL2*, *FOL3* and *DFR1* genes of folic acid biosynthesis pathway. The Multitemperature Single Strand Conformation Polymorphism (MSSCP) method and further nucleotide sequencing for selected strains were applied to indicate SNPs in selected gene fragments. The RT qPCR technique was also applied to examine relative expression of the *FOL3* gene. Furthermore, this is the first time ever that industrial yeast strains were analysed regarding genes of the folic acid biosynthesis pathway. It was observed that a correlation exists between the folic acid amount produced by industrial yeast strains and changes in the nucleotide sequence of adequate genes. The most significant changes occur in the *DFR1* gene, mostly in the first part, which causes major protein structure modifications in KKP 232, KKP 222 and KKP 277 strains. Our study shows that the large amount of SNP contributes to impairment of the selected enzymes and *S. cerevisiae* and *S. pastorianus* produce reduced amounts of the investigated metabolite. The results obtained here yield a list of genetically stable yeast strains which can be implemented as a starter culture in the food industry.

Key words: folic acid, *Saccharomyces cerevisiae*, gene polymorphisms, RT qPCR

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INTRODUCTION

Folate is a water soluble B vitamin, which is a cofactor in the one-carbon metabolism occurring in all cells. This vitamin is involved in many metabolic pathways, such as purine and pyrimidine biosynthesis, and amino acid conversions (Stover, 2009). Mammalian cells are unable to synthesize this compound and it is necessary to assimilate this vitamin exogenously. Its deficiency has been connected to a wide variety of disorders. One of the most widely discussed diseases are neural tube defects arising during fetal development (Rush 2000; Bestwick *et al.,* 2014). Furthermore, folate deficiency leads to elevated levels of plasma homocysteine, a risk factor for cardiovascular disease (Lonn *et al.,* 2006). Folate loss adversely affects DNA synthesis and cell division, and significantly reduces the efficiency of digestion and absorption of nutrients from the digestive tract. Too little supply of this vitamin leads to reduced concentration, to fatigue and even depression and anxiety. It is assumed that the body's need for folic acid, especially in the case of pregnant women, increases almost twice, therefore additional dietary supplements are required (McLone, 2003).

Plants and many microorganisms have the ability to synthesise folates through their folate biosynthesis pathway (Hanson & Gregory, 2011; LeBlanc *et al.,* 2013). The proper selection and use of folate-producing microorganisms is an interesting strategy to increase "natural" folate levels in foods. Folates included in meat products and vegetables are in the form of polyglutamine conjugates, which, prior to their assimilation, must be unraveled in the small intestine by deconjugates to monoglutamine, and then reduced to tetrahydrofolate and dihydrofolate. Yeasts are major producers of methylated polyglutamates (77.4%) and the unsubstituted polyglutamate form (tetrahydrofolate) (19.8%) (Seyoum & Selhub 1998), which are easily digestible (Martin 1983; Patring *et al.* 2005). It makes their bioavailability in the human cells much more significant than the synthetic counterparts of folic acid on the market (Pfeiffer *et al.* 1997). Therefore, in order to prevent the deficiencies, without taking supplements, it should be provided together with food. Many studies have shown that high intakes of folic acid, the chemically synthesized form, but not natural folates, can cause adverse effects in some individuals such as the masking of the hematological manifestations of vitamin B_{12} de-

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Abbreviations: *FOL2*, GTP-cyclohydrolase I gene; *FOL3*, dihydrofolate synthase gene; *DFR1*, dihydrofolate reductase gene; *TDH2*, triose-phosphate dehydrogenase gene; SNP, Single Nucleotide Polymorphism; MSSCP, Multitemperature Single Strand Conformation Polymorphism; RT qPCR, Real Time quantitative Polymerase Chain Reaction; TAE, Tris-acetate-EDTA buffer; TBE, Tris-borate-EDTA buffer; PAA, polyacrylamide

Table 1. Genes analyzed in this study

Gene	Enzyme	Conversion	Location
FOL2	GTP-cyclohydrolase I	first step of folate biosynthesis pathway	ChrVII: 1025735 →1025004
FOL3	dihydrofolate synthase	dihydropteroate → dihydrofolate	ChrXIII: 494999 → 496282
DFR1	dihydrofolate reductase	dihydrofolate → tetrahydrofolate	ChrXV: 780906 → 781541

ficiency, leukemia, arthritis, bowel cancer, and ectopic pregnancies (McNulty & Pentieva, 2004).

Saccharomyces cerevisiae and *Saccharomyces pastorianus'* ability to synthesise folates arises from their genetic apparatus. Folate content in different yeast strains displays large differences among strains that clearly indicate the importance of choosing the proper starter culture (Hjortmo *et al.,* 2008). In the biosynthesis of folic acid in yeast, the following major genes coding for various enzymes are involved (Table 1).

This is the first time ever when industrial polyploid strains of *Saccharomyces* were sequenced regarding the genes of folic acid biosynthesis pathway. So far, nobody else has carried out this type of research concerning yeast strains used in the industry, only strains of lactic acid bacteria have been investigated in this regard. Up till now, only a few researchers managed to obtain overexpression of two folate biosynthesis genes in wine yeast. This procedure resulted in elevated folate levels in wine only for the *FOL2* gene (Walkey *et al*., 2015).

The objective of this study was to select the optimal producing yeast strain by determining differences in nucleotide sequences in the genes of folic acid biosynthesis pathway. Our study revealed correlations between nucleotide sequence changes and amino acid composition in brewing and bakers' yeast strains. Moreover, RT qPCR analysis of the *FOL3* gene was performed to check the range of relative expression profiles among selected strains.

MATERIALS AND METHODS

Yeast strains and culturing media. Industrial yeast strains belonging to *S. cerevisiae* and *S. pastorianus* were used in the experiments. Strains were stored in liquid nitrogen and as lyophilized stocks with 10% skimmed milk at the Culture Collection of Industrial Microorganisms, prof. Waclaw Dabrowski Institute of Agricultural and Food Biotechnology. The twelve yeast strains used in the experiments were as follows: brewing yeasts (bottom and top fermentation) — KKP 201, KKP 219, KKP 211, KKP 222, KKP 223, KKP 192, KKP 183, KKP 217 and bakers' yeast — KKP 232, KKP 246, KKP 277. Yeast strains were cultured on Wort Agar plates (15 g l^{-1} malt extract, 0.78 g l^{-1} peptone, 12.75 g l^{-1} maltose, 2.75 g l^{-1} dextrin, 2.35 g l^{-1} glycerol, 0.75 g l^{-1} potassium dihydrogen phosphate, 1 g l^{-1} ammonium chloride, 1 g l^{-1} agar) (Merck Millipore, Schaffhausen, Switzerland) for 48h. Liquid precultures were prepared in 150 ml shake flasks containing 50 ml Wort Broth (15 g l^{-1} malt extract, 0.78 g l^{-1} peptone, 12.75 g l^{-1} maltose, 2.75 g l^{-1} dextrin, 2.35 g l^{-1} glycerol, 0.75 g l^{-1} potassium dihydrogen phosphate, 1 g l^{-1} ammonium chloride) (Merck Millipore, Schaffhausen, Switzerland), which were inoculated with a single colony from fresh plates and grown at 28°C in a rotating shaker, 100 rpm for 16 h.

Folate extraction and analysis. Folate extraction was prepared as described by Bagley & Selhub (2000) with modifications. Cells were spun down, the growth medium was discarded and pellets were transferred to 2 ml of a cold extraction buffer (50 mmol l^{-1} potassium tetraborate, 10 g l^{-1} sodium ascorbate, 2 g l^{-1} Triton X-100, pH 9.2), homogenized, and transferred immediately to a boiling water bath for 15 minutes. After boiling, the extract was cooled in an ice bath, neutralized with 0.4 ml of 1 mol l^{-1} monobasic potassium phosphate, and centrifuged at 4°C for 15 min at 14000 rpm. The supernatant fraction was stored at 4°C. Thus prepared samples were analysed using Immulite 2000 Folic Acid Kit (Siemens Medical Solutions, USA).

DNA isolation. Yeast cells were grown in 2 ml of Wort Broth for 48 h. The genomic yeast DNA extraction was conducted according to Amberg *et al.* (2005) with the following modifications**.** DNA pellets were washed once with 70% ethanol, air dried for 5 min and suspended in deionised water. DNA concentration was measured with NanoDrop ND-1000 (NanoDrop Technologies Inc. Wilmington, USA). Quality and purity of the DNA had to fulfil the criteria of 260/280 ratio $\geq$1.8 and 260/230 ratio $\geq$1.9. Integrity of DNA and the absence of RNA contamination were resolved with a 0.7% agarose gel. The DNA samples were stored at −20°C.

Primer design. Primers were designed to amplify the 295–470 bp region of the analysed genes as required by the MSCCP technique. S288C *Saccharomyces cerevisiae* strain was chosen as a reference to design primers using Oligo 6.68 and Primer3 software. Primers were purchased from Oligo.pl (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland).

PCR Reaction. Amplification reactions were performed in a 25 µl reaction volume containing 1x reaction buffer (100 mM KCl, 100 mM $(NH4)_2SO_4$, 200 mM Tris-HCl (pH 8.5), 20 mM $MgSO_4$, 1% Triton X-100); 0.2 pmol of dATP, dGTP, dCTP and dTTP, 0.2 pmol of each primer, 150–200 ng of DNA and 1U of the Run polymerase (A&A Biotechnology, Poland). Amplifications were performed in Hybaid Px2 thermal cycler (Thermo-Scientific, Waltham, USA) using the following primers and amplification conditions (Table 2 and Table 3).

To check the accuracy of designed primers, amplification products were separated by electrophoresis in a 1.5% (w/v) agarose gel with 0.5 TAE buffer.

MSSCP analysis. The PCR products were resolved by MSSCP (Multitemperature Single Stranded Conformation Polymorphism) for the genetic diversity of folate genes. The sensitivity of the MSSCP technique is generally inversely proportional to the size of the fragment (e.g. single base pair differences resolved 99% of the time for 100–300 bp fragments, >80% for 400 bp) (Kaczanowski *et al.* 2001). Each PCR reaction (2 µl) was diluted in 1.5 v/v denaturing solution B and 0.2 v/v solution A (Kucharczyk T.E.), denatured at 94ºC for 4 min, chilled on ice and resolved on a polyacrylamide gel. The electrophoresis was carried out in a vertical unit (MSSCP DNA Pointer, Kucharczyk T.E.) with Aplex 1006P power supply, in 5× TBE buffer. Sample resolution was carried out in 8% and 10% PAA gel (30% T, 3.3% C, 15% glycerol, Kucharczyk T.E.) or in 0.5 and 0.6 MDE™ Gel

Table 2. Primers used in this study

PCR Primer	Sequence (5′→3′)	Lenght (bp)	Amplicon (bp)	PCR Program
	FOL2			
FOL2F1	GGACCGCTGTAGGATGAAA	19	408	FOL2.1
FOL2R1	CCTTTTCCTCCTCTTCAGTTT	21		
FOL2U2	GGCCAAGTGTGGGTACAAGAC	21	390	FOL2
FOL2L2	TCGCATACATTTCTGCCAATC	21		
FOL2U3	CGGGTTAAGTAAGTTGGCCAG	21	391	FOL2.3
FOL2L3	AAGCTCAGTCGCGCGTGG	18		
	FOL3			
FOL3U	GGTCACCTGTTAGTAAGTTGATC	23	295	FOL3
FOL3_L1w	CTGGTATCTCTCTAACGGAATC	22		
FOL3_U2w	ACAATAAGCCGATTCCGTTAG	21	315	
FOL3_L2w	CCCCTTCTGTTATTATACCTGC	22		
FOL3_U3w	CAGGTATAATAACAGAAGGGGTA	24	298	
FOL3_L3w	GCCAATCTCGTACTTACTTCATTC	24		
FOL3_U4w	CAAGAATGAAGTAAGTACGAGATTG	25	470	
FOL3L	CACCACATAAATACAGTGAACCG	23		
FOL3Urt FOL3Lrt	GGTCACCTGTTAGTAAGTTGATC AACCTTTACCATTTGTCCCTG	23 21	161	RT qPCR
	DFR1			
ScDFRU	GCCGTTGCATTTGTAGTTTTTTCC	24	443	DFR
ScDFRLw1	CTCGTAGTTCGTTGCGCTCTTC	22		
ScDFRUw2	GAGAAATTGAAGAGCGCAACGAAC	24	392	
ScDFRLw2	CGTAGATTCTTTCCAGATGCTCC	23		
ScDFRUw3	GGAGCATCTGGAAAGAATCTACG	23	309	
ScDFRL	GCGGAGAGGTTTCATTTACGATTG	24		
TDH2Urt TDH2Lrt	GAACGATCCTTTCATCTCTAACGAC AGTCAATGGCGATGTCAATGTTTAG	25 25	194	RT qPCR

Solution (Lonza Rockland Inc.). The temperature profile of the electrophoresis was 30–15–5°C. Before applying samples onto the gel, 10 min pre-electrophoresis (40 W at 35°C) was performed. The samples were maintained for 10 min for concentration and then separated by MSSCP at 1000V/h for approximately 80 min. The separated ssDNA bands were visualized by silver nitrate staining (Silver Stain DNA Kit, BioVectis, catalogue number 200–101).

Product sequence. PCR products were chosen for subsequent Sanger sequencing (3730xl DNA Analyzer, Applied Biosystems, Carlsbad, USA) according to differences in patterns obtained with the MSSCP electrophoresis.

RNA extraction and cDNA synthesis. RNA extraction was performed using a commercially available kit (PureLink RNA Mini Kit, Ambion, Life Technologies). The yeast lysis was conducted using mechanical methods: yeast suspension was mashed with a pestle to release the intracellular lysate. Next, 500 µl of lysis buffer with 2-mercaptoethanol (10 µl ml^{-1}) was added to each sample and manufacturer's procedure was followed.

RNA quality and concentration were assessed spectrophotometrically and by electrophoresis in 1% agarose gels. The RNA samples were preserved at –80°C.

cDNA was synthesized with 50 ng μl^{-1} of previously diluted total RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) with oligo (dT) priming. This was followed by an RNAse treatment according to the manufacturer's instructions. The cDNA samples were stored at –80°C.

RT qPCR. RT qPCR analysis was performed in a Rotor Gene 6000 instrument (Qiagen) using the double-stranded-DNA-specific fluorochrome SYBR Green. Reactions were performed in a volume of 25 µl containing 1 µl of cDNA, 0.6 µl (10 µM) of forward and reverse primers and 12.5 µl of 2 × SYBR Green master mix (Maxima SYBR Green qPCR Master Mix, Thermofisher Scientific).

Priming temperature and RT qPCR programs were determined on the basis of temperature gradient tests. The reactions were run for 40 cycles with denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 17 s. An initial 10 min denaturation step at 95°C was used. In addition, product melting was assessed at the end of the reaction so as to verify the reaction specificity. Reactions conducted on the test samples afforded Cq values that described the formation of the product upon the assumption of a constant actual reaction yield. The threshold line was determined automatically by the software and the Cq values were

Table 3. PCR conditions

PCR Program	PCR conditions
FOL2.1	94°C – 4 min; 30 cycles of 94°C – 30 s, 51°C – 30s, 72°C – 35 s, 68°C – 15min
FOL2	94°C – 4 min; 12 cycles of 94°C – 30 s, 56°C – 30s, 72°C – 45 s, 23 cycles of 94°C – 30 s, 58°C – 30 s, 72°C – 45 s, 68°C – 15 min
FOL2.3	94°C – 4 min; 10 cycles of 94°C – 30 s, 58°C – 30s, 72°C – 40 s, 25 cycles of 94°C – 30s, 60°C – 30 s, 72°C – 40 s, 68°C – 15 min
FOL3	94°C – 4 min, 30 cycles of 94°C – 30 s, 58°C – 25s, 72°C – 30 s, 68°C – 15 min
DFR	94°C – 4 min, 30 cycles of 94°C – 30 s, 56°C – 30s, 72°C – 50 s, 68°C – 15 min

uploaded to an Excel file for analysis using the double delta method (Livak & Schmittgen 2001).

Statistical analysis. Statistically significant differences in mRNA level between analysed yeast strains were evaluated using one-way analysis of variance (ANOVA) and post-hoc Student's *t*-test. All calculations were performed using MS Excel sheet.

RESULTS

Folate biosynthesis in examined strains

The first stage of the experiment was to estimate the amount of folate biosynthesized by the cells of industrial *S. cerevisiae* strains. Summary of the results is presented in Table 4.

According to the conducted enzymatic tests, folic acid production was the least significant in brewing strains: KKP 192 and KKP 183, as well as bakers' strains: KKP 232 and KKP 277. The largest amount of this metabolite was produced by the strains of brewing yeast: KKP 201 and KKP 219. The increased biosynthesis of this metabolite can be also observed in the case of the bakery KKP 246 strain.

Searching for polymorphism using MSSCP analysis

In the first stage, the presence of genes responsible for encoding folate from the tested yeast strains was verified, as well as the accuracy of the primers designed for the experiment was determined by repeatedly performing PCR reactions. For each of the tested strain and gene fragment, the presence of products of the expected size was confirmed by agarose gel electrophoresis in the presence of an appropriate size marker. These products were then analyzed using the MSSCP method whose purpose is to detect mutations shown by variations in the system and configuration of electrophoretic bands in the studied gene fragments.

The *FOL2* gene was divided into three fragments and the following results were obtained: for the first part, four different polymorphic profiles; for the second part, the variability has not been demonstrated; for the third part two different sections were observed in the gel.

The *FOL3* gene, due to its length, was divided into four parts. As in the case of the *FOL2* gene, most changes were observed in the first part of the gene: it also shows four variables in the polymorphic profile. In the second part, the tested yeast strains showed no variation, while the third and fourth parts displayed two different patterns of bands in the gel.

Most of the changes concerning all the analyzed fragments of genes were observed after analysis of the first part of the *DFR1* gene — five variables in the band system were indicated, whereas as for the other genes analyzed, the second and third part displayed only two different profiles in the gel.

As one can see, based on the summary presented in Fig. 1 and Fig. 2, the most changes were observed in the DFRU_Lw1 and FOL3U_L1w fragments. These figures show the results of MSSCP electrophoretic separation, reflecting the changes in conformation of denatured ssDNA strands. Variable profiles are clearly visible in the case of the KKP 211, KKP 232 and KKP 217 strains (the *FOL3* gene), as well as KKP 211, KKP 222, KKP 192, KKP 232 and KKP 277 strains (the *DFR1* gene).

Table 4. Folate content in examined strains

No.	Species	Technological use	Strains (KKP)	Folate content (ng/ml)
1	*Saccharomyces pastorianus*	brewing yeasts	183	7.960 ± 0.12
2	*Saccharomyces pastorianus*		189	9.560 ± 0.23
3	*Saccharomyces pastorianus*		192	3.370 ± 0.11
4	*Saccharomyces pastorianus*		201	25.300 ± 0.41
5	*Saccharomyces cerevisiae*		211	21.400 ± 0.17
6	*Saccharomyces cerevisiae*		217	10.400 ± 0.11
7	*Saccharomyces cerevisiae*		219	23.700 ± 0.21
8	*Saccharomyces cerevisiae*		222	20.900 ± 0.18
9	*Saccharomyces cerevisiae*		223	20.200 ± 0.11
10	*Saccharomyces cerevisiae*	bakers' yeasts	232	5.400 ± 0.16
11	*Saccharomyces cerevisiae*		246	21.600 ± 0.41
12	*Saccharomyces cerevisiae*		277	8.660 ± 0.12

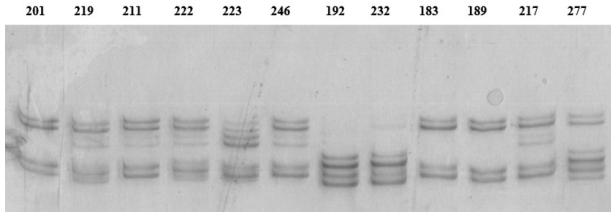

Figure 1. MSSCP of DFRU_Lw1 fragment for the KKP strains (201, 219, 211, 222, 223, 246, 192, 232, 183, 189, 217 and 277).

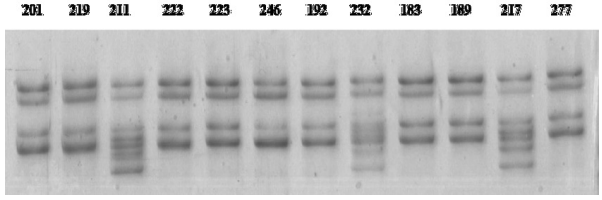

Figure 2. MSSCP of FOL3U_L1w fragment for the KKP strains (201, 219, 211, 222, 223, 246, 192, 232, 183, 189, 217 and 277).

The summary of variables within the electrophoretic profiles of gene fragments studied in the twelve tested strains is presented in Fig. 3.

Analysis of the *FOL2*, *FOL3* and *DFR1* gene sequences

In the next stage, appropriate gene fragments were selected and polymorphism was verified by using sequence analysis. For this purpose, a strain was selected as an internal positive control whose electrophoretic profile was the most reproducible among other strains — most often this was the KKP 201 strain. In addition, sequencing of the sample was representative of each of the variables concerning a polymorphic profile.

Gene fragments selected for sequencing for the different strains are shown in Table 5.

Subsequently, comparisons of DNA sequences in the tested strains were conducted, DNA sequences in the KKP 201 strain were selected as the internal control and the reference strain of *Saccharomyces cerevisiae* S288C was used. Summary of the changes is presented in Table 6.

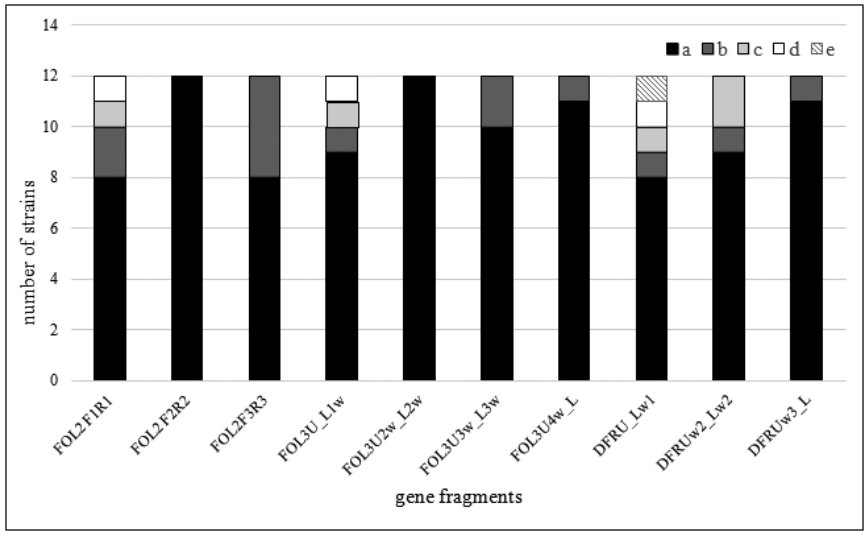

Figure 3 Differing amplification products (a, b, c d, e) among gene fragments in the 12 examined strains.

Table 5. Strains chosen for sequence analysis

Gene fragment	Strains chosen for sequence analysis (KKP)
FOL2F1R1	201, 223, 217, 277
FOL2F2R2	201
FOL2F3R3	201, 222, 192, 217
FOL3U_L1w	201, 211, 232, 217
FOL3U2w_L2w	201
FOL3U3w_L3w	201, 219, 217
FOL3U4w_L	201, 232
DFRU_Lw1	201, 211, 222, 192, 232, 277
DFRUw2_Lw2	223, 246, 192
DFRUw3_L	201, 192, 232

Most changes in the SNP character were present in the *DFR1* gene, which is responsible for the transformation of dihydrofolate to tetrahydrofolate, the active form of the folate. Most of the strains showed similarity to the S288C strain at the level of 99.7–99.1%. In case of KKP 277, KKP 192 and KKP 232 strains, more mutations were observed, mainly in the first part, and as a result the similarity to the reference strain was on the level of 98.7–98.8%. A relatively similar number of changes was observed in the *FOL2* gene at the level of 99.1–99.4%, and in the central portion thereof it was identical for all strains. The most stable gene of the tested yeast turned out to be *FOL3*, where single nucleotide changes (from 4 to 8) occurred only in the first part of the gene. Nucleotide alignments were created using the CLC DNA Workbench version 5.0.2 software program (CLC Bio USA, Cambridge, MA) and were verified manually.

Analysis of predicted amino acid sequences

On the basis of derived nucleotide sequences, the amino acid sequences were predicted using the CLC DNA Workbench version 5.0.2 (CLC Bio USA, Cambridge, MA) software. In according to nucleotide sequence mutations, amino acid changes also occurred. The most changes were observed in the DFRU_Lw1 fragment whose amino acid alignment is presented in Fig. 5. As shown in this figure, nucleotide changes caused major modifications of in dihydrofolate reductase structure in KKP 232, KKP 222, as well as in KKP 277.

Expression of the *FOL3* gene

For further RT qPCR research, the *FOL3* gene was selected, which is located on the chromosome VII, encoding the dihydrofolate synthase enzyme, responsible for the conversion of 7.8-dihydropteroate to 7.8-dihydrofolate in the folic acid biosynthesis pathway in *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* yeast. This reaction precedes conversion to the active tetrahydrofolate.

An experiment was conducted where the relative

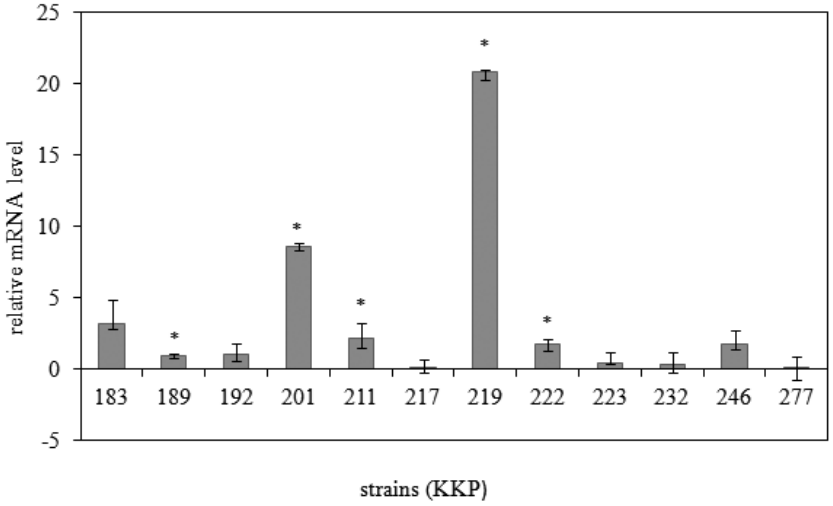

Figure 4. *FOL3* gene expression for the analyzed industrial KKP strains (183, 189, 192, 201, 211, 217, 219, 222, 223, 232 and 277).
*Indicates a statistically significant ($p<0.05$ in a Student's *t*-test) differences in mRNA level among strains.

expression of the gene in the analyzed strains was examined on the basis of the reference *TDH2* gene. The fragments amplified in the RT qPCR experiment were, according to the methodology, sequentially identical. Efficiency of the reactions was on the 98–99% level.

It has been shown that the highest level of expression, amounting to an increase in over 20 times, was characteristic for the KKP 219 strain. As a result, it exceeded the level of expression of the KKP 201 strain by two folds, where the relative expression level for KKP 201 was 8.63. Although a slightly higher folate biosynthesis was displayed by the KKP 201 strain, gene expression of *FOL3* in the conducted experiments was lower. This is probably caused by higher activityies of the other genes in the biosynthetic pathway, mainly *DFR1*, which is directly related to the metabolism of folate in a biologically active form. The same conclusions can be made for the KKP 211 strain – the level of expression of the analyzed gene (2.14) was incomparably lower to the synthesized folate level. The expression data for all yeast strains are shown in Fig. 4.

DISCUSSION

In 1931 Lucy Wills and her team had shown that preparations of autolyzed yeasts were an effective remedy in the fight against tropical, macrocytic anemia (similar to megaloblastic anemia) in the case of pregnant women in India (Wills *et al.,* 1931). The explanation of this phenomenon, as it turned out, was a high content of B vitamins in the applied formulation. Since then, folates began to be perceived as a new group of metabolites which are important to the proper functioning of the body.

The currently growing use of active microorganisms in the food economy (agriculture, environment-friendly methods of industrial and agricultural waste management, return to natural methods of production and conservation of food and feed), the most sought after microorganisms are the ones with specific properties, focused on production processes concerning functional foods (Gobbetti *et al.,* 2010; Kariluoto *et al.,* 2014). It is well known that, depending on the cultivation conditions and physiological state, the yeast can largely differ from each other as far as the phenotype is concerned (Hillenmeyer *et al.,* 2008; Bergström *et al.,* 2014). Experiments show that industrial strains of *Saccharomyces cerevisiae* are diverse in terms of biosynthesis of folates. In the experiments presented here, it has been demonstrated that there is a group of brewing and bakers' yeasts in which these properties are elevated.

It has been shown that the daily requirement for folate in the human diet oscillates around 400 μg/day. Studies show that the amount of this metabolite supplied with food is insufficient and that the daily demand is not catered for. Due to the risk posed by inadequate supply of this compound in food, in more than 70 countries, including the US, Canada and Australia, mandatory fortification of flour with mainly synthetic folic acid was introduced. Enrichment of flour with folic acid is fully justified, since it is a common and cheap source of food energy and the bread is an eagerly consumed product. Despite the evidence that such fortification is effective, over 120 countries have not introduced mandatory folic acid fortification, including all countries in the European Union. So far, no European country has decided to take such a step, although products of this type are becoming increasingly available and popular among consumers focused on the health-promoting lifestyle. EU policy tends more toward the marketing of functional foods than products with folic acid supplementation.

The difficulty that should be taken into account, while considering the possibility of folate food fortification, is their instability in technological processes. Numerous studies have shown that in chemical terms, folic acid is a compound undergoing degradation. Upon heating in acidic or alkaline environment, hydrolytic cleavage of the p-aminobenzoglutamate part follows, while in the neutral environment it is insignificant (McKillop *et al.,* 2002). Folic acid is also sensitive to light, oxidizing agents and reducing agents. In the aspect of supplementation of flour or bread, the most important seems to be the defense to overcome the loss of food products containing folate in the heat treatment process. These losses can go up to several tens of percent (Gujska & Majewska, 2005; Strandler *et al.,* 2015).

In the EU countries, supplementation of flour is not fully accepted also due to another reason: according to the experts, it may not be beneficial in some population groups. In accordance with some scientific reports, high doses of folic acid, especially in the case of the elderly, may mask the signs of anemia due to the fact that vitamin B_{12} deficiency plays a similar role in the body as folic acid, and ultimately leads to damage to the nervous system (Hirsch *et al.,* 2002). However, there is no such risk if folates are consumed naturally e.g. in the form of yeast biomass and its products (bread, beer, wine) (Hjortmo *et al.,* 2008) or in fermented dairy products (LeBlanc *et al.,* 2001). As is apparent from many reports, the best alternative method for increasing folate in food products is to use them in exactly the form mentioned above (Jagerstad *et al.,* 2005).

Table 6. Type of nucleotide changes in selected genes and strains

Gene	Strains (KKP)	Deletions	Insertions	Substitutions		The length of the analyzed sequence (bp)	Identity BLAST analysis (%)
				Transitions	Transversions		
FOL2	201	1	2	5	0	1189	99.3
	219	1	1	7	0		99.2
	211	1	1	7	0		99.2
	222	1	1	7	0		99.2
	223	1	3	5	0		99.2
	246	1	2	7	0		99.1
	192	1	2	5	0		99.3
	232	1	2	5	0		99.3
	183	1	2	5	0		99.3
	189	1	2	5	0		99.3
	217	1	1	5	0		99.4
	277	1	2	5	0		99.3
FOL3	201	0	0	3	1	1379	99.7
	219	0	0	2	1		99.7
	211	0	1	4	1		99.5
	222	0	0	3	1		99.7
	223	0	0	3	1		99.7
	246	0	0	3	1		99.7
	192	0	0	3	1		99.7
	232	0	2	5	1		99.4
	183	0	0	3	1		99.7
	189	0	0	3	1		99.7
	217	0	1	2	1		99.7
	277	0	0	3	1		99.7
DFR1	201	6	2	1	0	1144	99.2
	219	6	2	1	0		99.2
	211	6	2	1	0		99.2
	222	6	3	1	1		99.1
	223	6	2	1	0		99.2
	246	0	2	1	0		99.7
	192	0	2	8	3		98.8
	232	6	3	3	2		98.7
	183	6	2	1	0		99.2
	189	6	2	1	0		99.2
	217	0	1	5	1		99.3
	277	6	6	1	0		98.8

Folates supplied in the diet, together with meat and vegetables, are in the form of polyglutamic conjugates that have to be distributed in the small intestine by decognase to monoglutamics, which are then reduced to tetrahydrofolate and dihydrofolate. Folate produced by the yeast represents the form of easily assimilated monoglutamic compounds, and thus makes its bioavailability much higher (Halsted, 1980). Properly used yeast strains with high ability to synthesize folates, applicable in biotechnological processes, do not require any additional financial contribution for their use.

Accurate knowledge of the genetic structure of industrial strains of the *S. cerevisiae* yeast is the primary criterion for the selection and choice for a biotechnological process (Misiewicz, 2013).

Studies on the biosynthesis of folic acid conducted by researchers supervised by Hjortmo (2005) confirmed that these microorganisms are among the most efficient producers of folic acid. 100 g of dry weight ranged in containing 4000 to 14500 μg of folate, and compared to a control strain of bakers' yeast, folate content was more than 2 times higher. In addition, most scientists

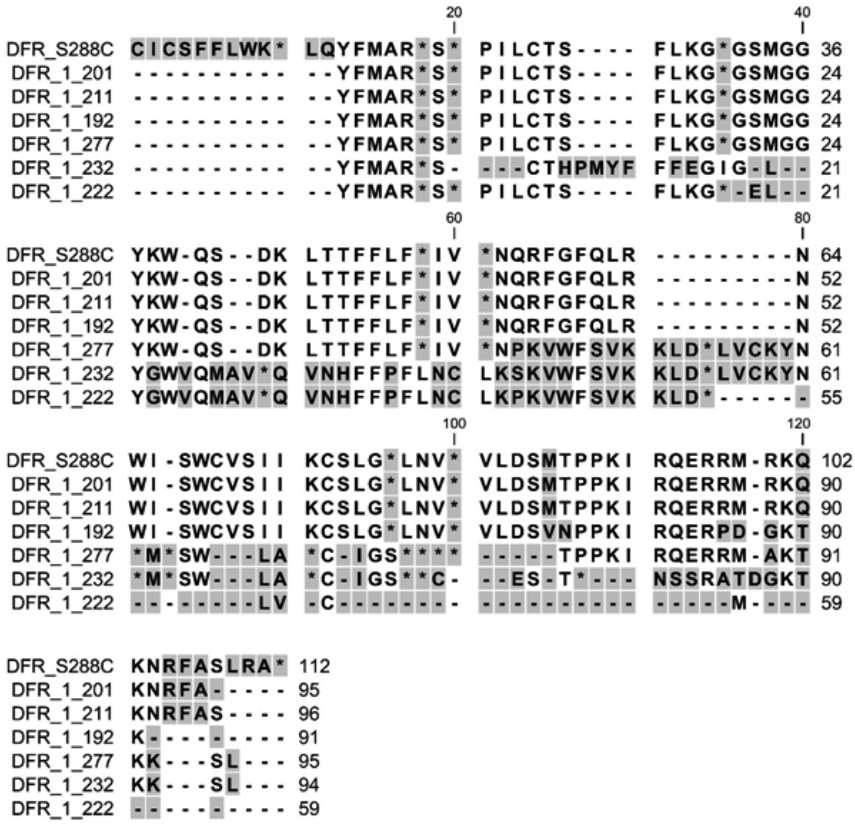

Figure 5. Amino acid sequence alignment of the DFRU_Lw1 fragment for the S288C reference strain and the analyzed KKP strains (201, 211, 192, 277, 232, 222).

note that technological processes, such as fermentation, are the only ones that do not cause losses, and additionally enable increasing the concentration of folate in food. For example — while ensuring that appropriate cultivation conditions and choosing the right strain of yeast, the starting order of folate biosynthesis 27–43 μg/100 g of bread was increased to 135–139 μg/100 g (Hjortmo *et al*., 2008). Research conducted by the team of Jegerstad (2005) argued that the alcoholic fermentation carried out by yeast, or the use of strains of the *Lactobacillus* lactic acid bacteria raises folate levels by two and three times in products such as bread, vegetables, beer and dairy products. Increasing the folate content by yeast has already been demonstrated in many fermented raw materials: rice leaven and traditional products (Kariluoto *et al.,* 2006; Hjortmo *et al.,* 2008), raw grain (Kariluoto *et al.,* 2014), dairy products (kefir) (Patring *et al.,* 2006).

It has been shown that folate content of commercially available beers fluctuates within the range of 2.2 to 24.2 μg which represents only 6.1% of the daily requirement of this component (Owens *et al.,* 2007). Recent reports in the professional literature show, however, that using wine yeast strains can, through genetic engineering, raise folate content more than 2 to 4 times in the final product which is wine (Walkey *et al.,* 2015). Until now, it has been demonstrated that homologs of the *FOL2* gene, encoding the GTP cyclohydrolase I, were successfully used in other organisms to boost folate levels (Nardese *et al.,* 1996; Mancini *et al.* 1999). In tomatoes and *Arabidopsis*, over-expression of the homologous gene results in a two- to four-times increase in folate levels (Diaz the la Garza *et al.,* 2004; Storozhenko *et al.,* 2007).

The molecular basis of the technological properties of industrial yeast are still unknown. The obvious advantage of this type of yeast, as compared to non-laboratory strains, is their polyploidy and hydrophobic properties and continuously generated polymorphism. Such construction suggests their ability to adapt and their genome plasticity (Misiewicz & Wroblewska-Kabba, 2013). Many synonymous substitutions shown in available studies show that transitions occur more frequently than transversions. According to the proponents of neutral theory, mutations are the dominating force in differentiating species among themselves, and most of the differences between the species are a result of accidental consolidates of nearly neutral mutations in the second and third generation (Misiewicz, 2013). It was confirmed, that subtelomeric regions variability allows yeasts adaptation to different carbon sources (Winzeler *et al.* 2003; Schacherer *et al.* 2007). Some cases have demonstrated the sequence polymorphism influence on the industrial phenotype (Guillaume *et al.* 2007; Galeote *et al.* 2010). Lower number of SNPs in *DFR1, FOL2* and *FOL3* genes in the wine yeast strains when compared to the SNPs in subtelomeric genes, as in the *FLO* family, results from their localization, much closer to centromere (Misiewicz, 2013).

In order to verify whether the mutations in a specific gene have an influence on the structure of the encoded protein, an extensively discussed method of intermediate genotyping by PCR-MSSCP is used. The method is based on detecting changes in electrophoretic mobility of single-stranded DNA formed by conformers (Walker & Rapley 2005). This technique has been already often used with success, among others, to differentiate the individual genetic profiles of 26S rDNA of yeast involved in the process of fermentation (Duarte & Baleiras-Couto, 2012), or identifying unknown spe-

cies of fungi through electrophoretic differentiation of 18S and 28S rRNA subunits and the ITS regions (Janke *et al.,* 2013). The method used is based on the assumption that mutations occurring within the analyzed fragment trigger a conformational change of ssDNA which could change the rate of migration in a polyacrylamide gel (Kerr & Curran, 1996). Thanks to this system, it is possible to detect minor mutations at the level of about 80–90%, with the best results obtained when the length of the strand does not exceed 300 bp (Hayashi & Yandell, 1993). Fragments of this size were used for the analysis of the first, second and third parts of the *FOL3* gene and a third part of the *DFR1* gene. With increasing length of the fragments examined, the effectiveness of the method theoretically decreases, although this cannot be unambiguously measured, as it depends on many factors. In some studies, detection at the level of 84% in analyzing fragments of 450 bp length or even greater was obtained. In the case of long pieces of sequence, a single mutation cannot raise the electrophoretic mobility shift, though conformation may change (Jordanova *et al.,* 1997; Sunnucks *et al.,* 2000). A substantial role in the efficacy of the method used is played by the temperature that significantly affects the stability and structure of the secondary DNA strand (Kaczanowski *et al.,* 2001; Szewczyk *et al.,* 2008; Tomczyk-Zak et al., 2012). Using changes of temperature during electrophoresis in 15-20% of the aforementioned studies increased the chance of distinction of two different conformations. The optimum temperature and other conditions of separation were selected experimentally. Each molecule of ssDNA is converted, so as to obtain a spatial structure that provides with a free energy minimum, however, one should take into account the fact that the energy difference between conformers may be minimal, which predisposes the formation of sub-conformers under the same conditions. When reading the gel, this phenomenon is seen as the appearance of several bands and is viewed as desirable, as a change of even one band indicates a change in the sequence (Kaczanowski *et al.,* 2001). Such phenomenon is very noticeable in the first part of the *DFR1* gene. In the image of the polyacrylamide gel, the fragment of each strain has several bands.

Comparing the results concerning the level of natural folic acid produced in the yeasts, with the results of research involving genetic polymorphism in the *FOL2*, *FOL3* and *DFR1* genes, it has been shown that mutations in these genes result in damaging the formation of a fully functional enzyme. Sequencing of fragments of genes in strains whose band pattern in MSSCP was different from the adopted internal control, confirmed the high genetic variability. Differences in the genetic structure of these strains may have caused formation of a non-functional enzyme because the biosynthesis of folic acid in these strains is at a reduced level. The most visible correlation can be seen in the KKP 232 and KKP 277 strains.

On the other hand, high folate biosynthesis was demonstrated in the brewing yeast KKP 219 strain. Out of all tested strains, this one had shown an above-average productivity, as well as genetic stability within the tested genes. In most cases, the pattern of bands in the MSSCP analysis was identical to the reference strain. In addition, the relative expression level of the *FOL3* gene in this strain was the highest one among the analyzed strains. The results obtained here confirm the possibility of predicting these properties by using molecular methods that enable rapid selection of industrial strains. This study shows that the differences of an SNP character affect the amino acid sequence of the encoded enzymes which might give rise to an enzyme which is not performing its function efficiently. Most changes of a frame shift character were demonstrated by the *DFR1* gene, which encodes a crucial enzyme allowing the conversion of folate to the active form. Mutations in this gene contribute to reduced productivity of the folate. The obtained results suggest that the KKP 219 and KKP 201 strains are the most efficient producers of folic acid within the twelve examined strains.

The use of natural industrial strains that are genetically stable and display an above-average folate biosynthesis may be a promising way to produce food without the use of genetic engineering, which is presently causing much controversy. Consumers are increasingly afraid of GMO, avoid processed foods and tend to choose the natural production methods.

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