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AQUACULTURE AND AGRICULTURE BIOMASS SIDE STREAM PROTEINS AND BIOACTIVES FOR FEED, FITNESS AND HEALTH PROMOTING NUTRITIONAL SUPPLEMENTS	
Grant agreement:	790956
Duration:	April, 2018 – December, 2022
Coordinator:	NOFIMA AS, Norway

PROJECT-BASED LEARNING

Bioactivity assays with S. cerevisiae model cultures grown on processed fish side-streams

Small Research Project

'Study the main adaptive indicators (central carbon metabolism and ROS homeostasis)'

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the BBI grant agreement No 790956

<table>
<tr><td>Project title</td><td colspan="3">Bioactivity assays with S. cerevisiae model cultures grown on processed fish side streams – study the main adaptive indicators (central carbon metabolism and ROS homeostasis)</td></tr>
<tr><td>Responsible Organization(s)</td><td colspan="3">R&D Center Biointech Ltd</td></tr>
<tr><td>Project duration</td><td colspan="3">5 months</td></tr>
<tr><td rowspan="2">Research Effort Categories (%)</td><td>Basic</td><td>Applied</td><td>Developmental</td></tr>
<tr><td>10</td><td>90</td><td>-</td></tr>
<tr><td rowspan="2">Background data</td><td>Field of science</td><td>Knowledge area</td><td>Subject of investigation</td></tr>
<tr><td>Biology/Biotechnology</td><td>Microbial growth on fish side streams</td><td>Metabolic response and adaptation mechanisms</td></tr>
<tr><td>Summary</td><td colspan="3">This project is focused on the appraisal of yeast growth on non-traditional carbon and energy sources based on fish by-product hydrolysates as an indicator of cellular adaptation. The changes in central carbon metabolism are studied in the model S. cerevisiae, grown on the specified nutrients through spectrophotometric activity measurement and Multi-Locus Enzyme Analysis of main enzymes from the Glycolytic pathway, Krebs cycle, and Pentose-phosphate pathway. The data obtained indicated a similar range of activities of the main enzymes of the sugar metabolic pathways and uniform enzymes pattern in these cultures. The ROS (Reactive Oxygen Species) homeostasis was evaluated in the model culture through the characterization of the major components of the antioxidant defense system: superoxide dismutase (SOD) and catalase (CAT) activities, and glutathione level. The results observed indicate that the cells have evolved a balanced mechanism to neutralize the extra ROS, namely the enzymatic and non-enzymatic antioxidants, which collectively reduce the oxidative state.</td></tr>
<tr><td>Project objectives</td><td colspan="3">1. Study the changes in central carbon metabolism metabolic changes tracking the activities of the Glycolytic pathway, TCA, and Pentose-phosphate pathway essential enzymes;
2. Investigation of the cellular redox homeostasis in S. cerevisiae through characterization of the main components of the antioxidant defense system - an indicator for the substrate utilization efficiency.</td></tr>
<tr><td>Project Methods</td><td colspan="3">Along the project, the following methods and techniques will be exploited:
- Batch cultivation technique;
- Biochemical analyses:
o Cell-free extracts preparation;
o Determination of protein concentration;
o Biochemical methods for investigation of enzyme activities: Hexokinase (EC: 2.7.1.1.), Glucose-6-phosphate dehydrogenase (1.1.1.49.), 6-Phosphogluconate dehydrogenase (1.1.1.44.), Isocitric dehydrogenase (NAD/P) (EC 1.1.1.42.), Glyceraldehyde-3-phisphate dehydrogenase (EC 1.2.1.12.), Succinate dehydrogenase (1.3.99.1), Fumarase (EC 4.2.1.2.), Superoxide dismutase (EC1.15.1.1.), Catalase (1.11.1.6.), Total glutathione.
- Multi-locus enzyme electrophoresis analysis: Glucose-6-phospahe dehydrogenase, 6-phosphogluconate dehydrogenase, Isocitrate dehydrogenase (NAD/NADP).</td></tr>
</table>

Accomplishments under project objectives	
Work performance Monitoring the enzymes activities of Glycolysis (Embden-Meyerhof-Parnassus pathway), Tricarboxylic Acid Cycle (CAC) and Pentose-Phosphate pathway.	**Results:** - Glycolysis. The enzymes activity of hexokinase (EC: 2.7.1.1.) and glyceraldehyde-3-phosphate dehydrogenase (EC: 1.2.1.12.), essential for glucose uptake through glycolysis, were monitored. The results are shown in Fig. 1 A and B. The phosphorylated glucose (due to hexokinase activity) is excreted difficultly from the cell. On the other hand, in addition to its basal function (phosphorylation), under certain conditions, glyceraldehyde-3-phosphate dehydrogenase initiates cellular apoptosis and participates in the antioxidant protection of the cell. In the presence of elevated ROS levels, the enzyme activity is inhibited, and the catabolisation of glucose starts via the pentose-phosphate pathway. As a result, the NADPH, necessary for the functioning of the antioxidant enzymes glutathione reductase and thioredoxin reductase, increases. – Pentose-phosphate pathway. The activities of the main regulatory enzyme of the pathway - glucose-6-phosphate dehydrogenase (EC: 1.1.1.49.), together with the enzyme 6-phosphogluconate dehydrogenase (EU: 1.1.1.44.) were measured (Figs. 4 A and B). A trend for relatively low values measured was established. The reason for these results lies in the specific role the enzymes, glucose-6-phosphate dehydrogenase in particular, in cellular metabolism. This enzyme is responsible for the relationship between glycolysis and the pentose-phosphate pathway, performing a regulatory function in directing metabolic reactions to catabolism (glycolysis) or anabolism (pentose-phosphate pathway). In addition, the two enzymes studied are responsible for the production of reduced equivalents, maintaining the NADPH/NADP ratio at relatively constant high levels that ensure the performance of glutathione reduction/oxidation reactions under conditions of oxidative stress. For this reason, they are also considered essential enzymes involved in the antioxidant protection of the cell. – Three-Carboxylic Acids (Krebs) Cycle. The enzymes isocitrate dehydrogenase (NAD (EU: 1.1.1.41.) and NADP-dependent (EU: 1.1.1.42.)), fumarase (EU: 4.2.1.2.), succinate dehydrogenase and fumarase were analysed as markers of mitochondria and electron transport chains functioning. The data (Figs. 5 A and B and Figs. 6 A and B) show that these enzymes exhibited behaviour and specific activities similar to the glycolysis enzymes. The increased metabolic activity of the yeast cells reflects the active transcription and translation of proteins encoded by genes from central metabolism and enhances protein synthesis. - Multi-Locus Enzyme Analysis (MLEA). Three main enzymes of carbon metabolism, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and NAD/NADP isocitrate dehydrogenase, were subjected to MLEA. The number of isoforms and their relative mobility (Rm) is outlined in Table 2. The presented data indicate that the number of enzyme isoforms and their Rm values for glucose-6-phosphate dehydrogenase were similar in all tested cultures. The gene *ZWF1,* which codes for the enzyme glucose-6-phosphate dehydrogenase, is expressed as two NADPH-

	dependent forms localized in the cytoplasm. The same pattern was observed for the enzyme 6-phosphogluconate dehydrogenase: 2 isoforms that exhibit similar Rm values for all tested cultures. The electrophoretic profile of the NAD-dependent isocitrate dehydrogenase revealed the expression of isoenzymes, apparently products of the *IDH1* и *IDH2* genes found in *S. cerevisiae* yeasts. As regards the NADP-dependent form of the enzyme, three isoforms were registered in all tested cultures, encoded by the respective *IDP* genes. **Conclusions:** - The summarized data indicate that in addition to the similar range of activities of the main enzymes of the sugar metabolic pathways, the MLEA confirmed the uniform enzyme pattern in these cultures.
Work performance Characterization of major components of the antioxidant defence system in *S. cerevisiae*:	**Results:** - Measurement of reduced and oxidized glutathione. The amount of reduced and oxidized glutathione in the cells of the *S. cerevisiae* model strain grown on the tested media was assayed to establish the role of glutathione in the antioxidant protection of the cell. The reduced and oxidized glutathione levels were lower in the processed side streams compared to the control sample (Table 3). This reduction is most likely due to the fact that, although reduced glutathione (GSH) is one of the main antioxidant molecules in the cell, and is claimed to play an essential role in cellular defence against ROS, the significant supply with SH-containing free amino acids may undertake the detoxifying action of this tripeptide resulting in lower GSH activity in the processed side-stream media. - Antioxidants enzymes activities assessment. The role of the main antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) in protecting the yeast cells against oxidative damage was evaluated. The comparative analysis between the total SOD activity measured in the control culture and those grown on processed side streams showed that in the latter, the expression of this enzyme increased significantly (Table 3). As regards the specific CAT activity, it elevates in the processed side-stream media. This increase is likely due to the fact that the principal biological role of CAT A is related to the disposal of H_2O_2 formed in the processes of β-oxidation of fatty acids. On the other hand, CAT T is engaged in various stress conditions protection, such as thermal and osmotic shock, starvation or high concentrations of H_2O_2. **Conclusions:** - Cells have evolved a balanced system to neutralize the extra ROS, namely antioxidant systems that consist of enzymatic antioxidants such as SOD, CAT, and glutathione peroxidases (GPxs), thioredoxin (Trx) as well as the non-enzymatic antioxidants, which collectively reduce the oxidative state. - The efficient synthesis of *S. cerevisiae* biomass enriched in antioxidant enzymes on the processed side-stream media tested, represents a good opportunity for its use in various industrial applications.
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RESEARCH DATA

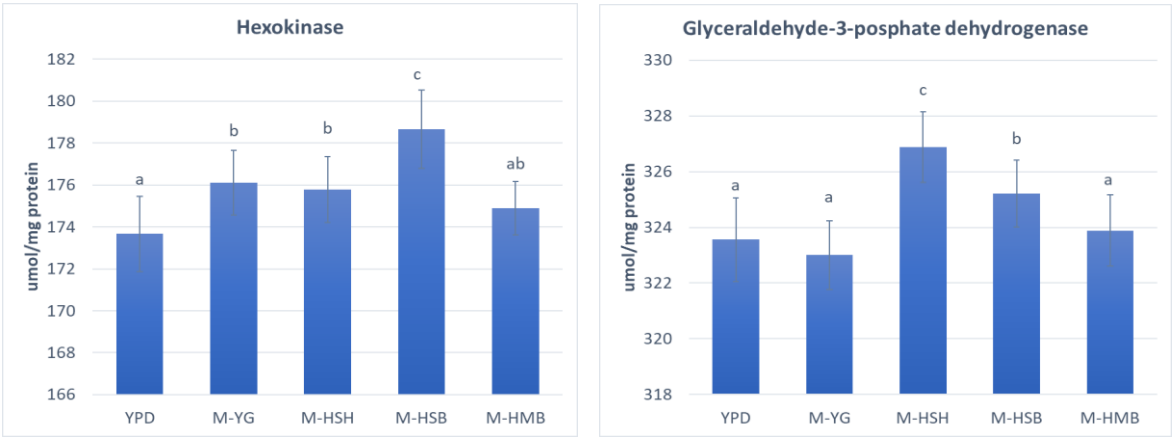

Figure 1. Activities of key glycolysis enzymes; A – hexokinase; B - glyceraldehyde-3-phosphate dehydrogenase; $p < 0.05$

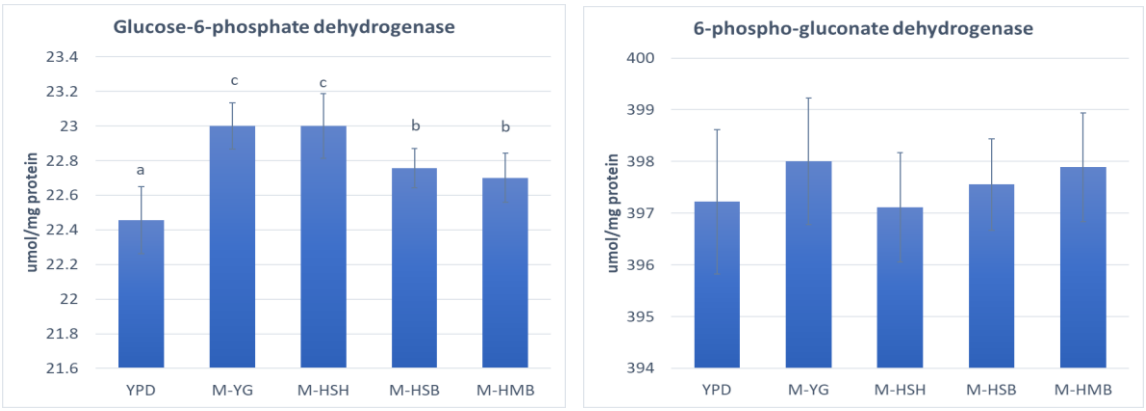

Figure 2. Activities of pentose-phosphate pathway key enzymes; A – glucose-6-phosphate dehydrogenase; B – 6-phospho-gluconate dehydrogenase; $p < 0.05$

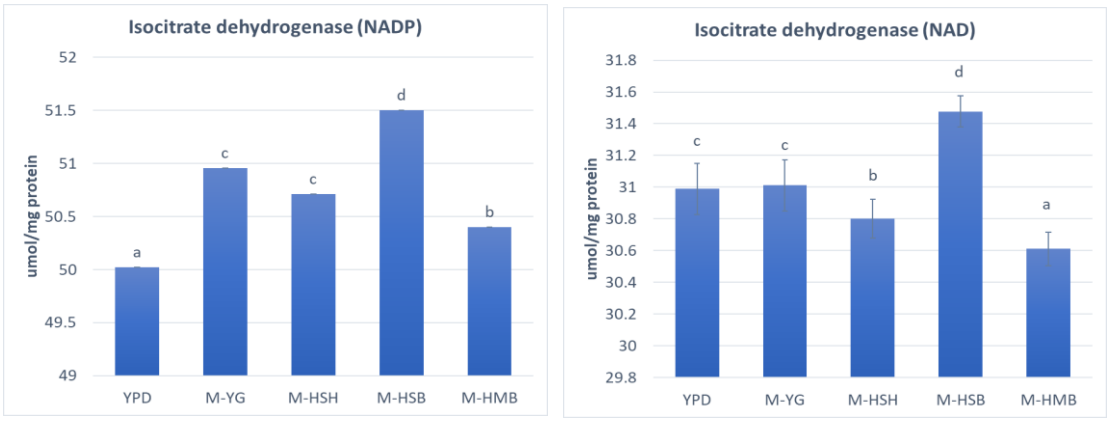

Figure 3. Activities of Krebs cycle key enzymes; A – isocitrate dehydrogenase (NADP); B isocitrate dehydrogenase (NAD); $p < 0.05$

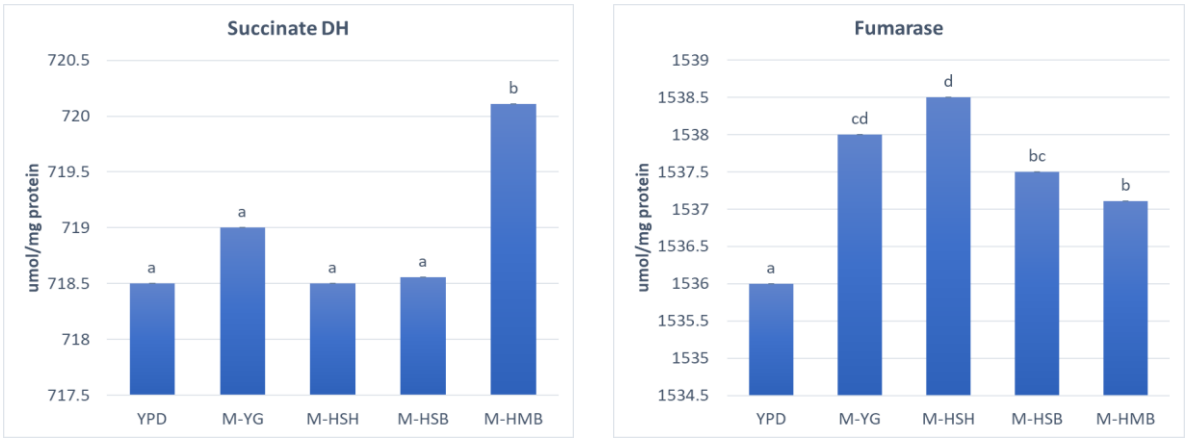

Figure 4. Activities of Krebs cycle key enzymes; A – succinate dehydrogenase; B – fumarase; $p < 0.05$

Table 1. MLEA of key enzymes of C-metabolism; $p < 0.05$

Enzyme	Nr of isoforms	Rm values
Glucose-6-phospahe dehydrogenase ➢ M-HSB ➢ M-HSH ➢ M-HMB	 2 2 2	 0.186±0.007; 0.349±0.02 0.184±0.006; 0.348±0.02 0.185±0.006; 0.349±0.03
6-phosphogluconate dehydrogenase ➢ M-HSB ➢ M-HSH ➢ M-HMB	 2 2 2	 0.260±0.01; 0.341±0.03 0.263±0.01; 0.343±0.03 0.261±0.01; 0.343±0.02
Isocitrate dehydrogenase (NAD) ➢ M-HSB ➢ M-HSH ➢ M-HMB	 2 2 2	 0.172±0.008; 0.257±0.01 0.171±0.009; 0.256±0.01 0.170±0.009; 0.256±0.01
Isocitrate dehydrogenase (NADP) ➢ M-HSB ➢ M-HSH ➢ M-HMB	 3 3 3	 0.170±0.008; 0.288±0.03; 0.316±0.02 0.171±0.008; 0.285±0.03; 0.312±0.02 0.172±0.007; 0.286±0.03; 0.313±0.01

Table 2. Characterization of the main components of the antioxidant defence system in *S. cerevisiae* strain; $p < 0.05$

Medium	SOD (U/mg of protein)	Catalase (U/mg of protein)	Total glutathione (mM/mg of protein)
M-HSH	51.5 ± 0.6	26.11 ± 0.7	0.42 ± 0.01
M-HSB	55.0 ± 0.9	21.40 ± 0.8	0.42 ± 0.01
M-HMB	50.0 ± 0.4	23.64 ± 0.7	0.49 ± 0.02

LEARNING BEENFITS

Learning Outcomes	After successful accomplishment of project activities, the trainees are able to:		
	Knowledge - Describe biochemical investigations with yeasts - Match experimental data and theoretical considerations - Define approaches for bioactivity assays arrangement	Skills - Apply enzymological methods - Demonstrate techniques for microbial bioactivity assays performance - Use specialized equipment	Autonomy/responsibility - Collaborate with colleagues - Carry out tasks independently - Present and report research data

Outputs / Impact	
Target audience trained	This project is foreseen for: - Graduates and Postgraduates (MSc, PhD students) - Post-doctoral Researchers and Research Associates - Academic professionals (Tutors)
Research results disseminated to communities of interest	Distribution of project results at intra-institutional and intra-partnership level
Opportunities for training and professional development	The benefits for the project targets: - New specific knowledge and skills acquired - Problem/solution-based thinking mastered - Team-working abilities enhanced - Understanding of circular economy principles clarified