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## THE INFLUENCE OF CHROMIUM COMPOUNDS ON YEAST PHYSIOLOGY\*

(A REVIEW)

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### Introduction

Metal ions and yeasts metabolism was reviewed quite a few times in the last decades by eminent researchers [1–4]. In spite of this excellent work in the past it is clear that metal ions nutrition in yeasts is still open for further research. After specifying requirements and functions of macroelements in yeasts [4, 5] the era of trace

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elements [6] started and in the recent years one can see many publications considering the problem of trace elements, which seems to be really needed for efficient metabolism. It seems that new analytical approaches [7] busted new wave of research (i.e. NMR, EPR, Raman spectroscopy) into the field of ionic nutrition, which was already in the focus some years ago [6, 8, 9].

The problem of ionic nutrition is also strongly oriented to the human diet. Regarding this topic there are profound reviews available [10, 11] and some of them specially emphasize chromium [12, 13, 15], since it became important through revealing its function in glucose tolerance factor (GTF). Consequently, all the results from studies of ionic nutrition in microbes are oriented towards metabolism of higher organisms and finally to human diet [16, 17]. It is evident that nutrition of human is becoming one of the major fields of research [18] with laying stress on understanding metabolism [19], defining standards of mineral intake [20] and creating functional foods [21, 22].

In the environmental field one can see significance of metal ions in two directions: the pollution control and mining technology. The removal or uptake of metal is concerned with the increasing value of some metals on one hand, and with awareness for ecological effect of toxic metals released into the environment on the other [3, 23–26]. In the area of environmental care the conventional methods for removing metals from polluted streams or ore processing solutions can include traditional chemical and physical processes which can be ineffective or extremely expensive, especially when metals are present in low concentrations from 1 to 100 µg/mL [27, 28]. In addition, the history of the waste (source and its pretreatments) seems to play an important role for selecting proper microbial biomass for biosorption with a goal of bioremediation. In some cases it was proved that although the yeast biomass was capable of removing more than 99% chromium from electroplating wastewater, it was only capable of removing 50–60% of chromium from tannery wastewater [29].

Regarding environment we should be very careful since everything what was released into soil, water or air from technological activities comes back to food chains and consequently – directly or indirectly – to human diet [30]. The link to environmental biochemistry of chromium is evident [31] what is crucial for tracing chromium flux in environmental niches and understanding oxidation-reduction dynamics in chemical [32, 33] as well as in biological world [34].

Moreover, successful ionic nutrition is related to the metal uptake, which is understood as biosorption and bioaccumulation and is important for all living creatures. Selective advantages are confirmed for species which are capable of uptake and retaining ions (from metal or transition elements) available in limited amounts in

surroundings. During the last decades the trace elements such as Mn, Fe, Co, Ni, Zn, V and Cr, were found to be essential or at least beneficial for normal metabolism in all organisms [35].

The metal uptake starts with fast biosorption on the cell wall and then continues with slower bioaccumulation. Bioaccumulation mechanisms consist of metal ion translocation through the cell membrane into the cell and its deposition in adequate store compartment in living cell. In principle, all the steps mentioned are metabolically dependent, what is not the case in metabolically independent passive processes, which are based on different mechanisms for metal sequestering onto the cell surface. Biosorption is strongly affected by combination of other metal ions in cell surroundings [36]. This uptake is metabolically independent but depends on the chemical make-up of the cell wall of particular species. Enormous diversity among yeasts has been found by various approaches of studying yeast cell envelope [37]. Consequently, the metal type, its ionic form in particular solution, and structure of an active binding site responsible for sequestering the metals are factors which should be included into the predicted model when influence on biosorption is concerned. It depends on the degree of affinity between different types of metal species or its ionic forms and the target site at the cell wall molecular structure [38]. Additionally, competition of other ions should not be ignored since there are some observations showing competition effects (i.e. in cadmium/iron system) [39].

Bioaccumulation is generally defined as active mode of metal uptake by living cells [3] and depends on its metabolic activity. Bioaccumulation process can be significantly affected by the presence of metal ions, which are accumulated. Additionally, the tolerance of yeast to the environmental concentration of metal ion is crucial for conducting bioaccumulation. Consequently, yeast ability to control the intracellular concentration of the ion in the range, which is not toxic for metabolic processes, have to be maintained for efficient bioaccumulation.

When a selection of suitable microorganism for metal bioaccumulation is in consideration, the yeasts showed to be advantageous among the microbial candidates, as they have been known to serve human needs for a long time. They can be exploited in metal removing processes from industrial wastes [40], as well as for human and animal nutrition since they belong to microbes which are traditionally recognized as safe [41]. However, the status of safe yeast is dominantly adopted for *Saccharomyces cerevisiae*, which is the common species present in food and feed production as well as in human and animal diet.

Nutrition has been very important in the last years in human and animal diet [11]. Mineral nutrition is one of the key issues in functional foods [21]. As an example of food mineral nutrition chromium can be selected. The chromium function as the key

element in glucose tolerance factor has been studied from different perspectives since 1957 [42]. In respect to chromium metabolism and function there are still many nonclarified situations, which additionally stimulate research in bioaccumulation. Majority of publications claims that it is an essential element for glucose and fat metabolism in mammals [12, 14, 16, 43–45]. There are not many informations about chromium tolerance, ionic nutrition [46], transport [2], biosorption [47, 48] and bioaccumulation in yeasts [49–51]. The natural chromium content in *Saccharomyces cerevisiae* is less than 0.07 µg/g dry mass [49], when its availability depends on natural substrates for growth. It is claimed that its structural function is the stabilization of the tertiary structure of proteins or nucleic acids on one hand and the metabolic function in glucose metabolism in mammals [12, 14] and yeasts [52–54] on the other hand.

### Chromium as an ion of choice for yeast metabolism

Chromium is included on the list of essential trace elements for humans and some animals [45] and expresses at least beneficial effect in other living species tested including yeasts [52]. Depending on its form it can be toxic and even cancerogenic [55, 56].

Chromium belongs to the first series of transition metals. Its position in subgroup VI B of the periodic system is surrounded by three elements with a known biological function, like vanadium, manganese, and molybdenum. Chromium can occur in every oxidation state from 2– to 6+, but the most commonly found are 0, 3+, and 6+ [34]. The 2+ state does not exist in the biological system [12, 14, 44]. Tetravalent and pentavalent chromium oxidation states are rare and unstable, like divalent chromium. However, the electron precession resonance (EPR) spectroscopy has shown stabilization of the pentavalent complexes, formed via the reduction of hexavalent chromium with various biological thiols prior to DNA binding [57]. The hexavalent form is almost always linked to oxygen, and forms chromate ( $\text{CrO}_4^{2-}$ ) or dichromate ( $\text{Cr}_2\text{O}_7^{2-}$ ) ions, which are strong oxidants. The hexavalent chromium compounds demonstrate acidic properties and their colors range from yellow to orange [12, 14, 57]. This ionic complex has negative charge what makes it very suitable for transport by sulfate transporters into the cell. These ions are easily reduced to Cr(III) in acidic environment. Additionally, the presence of reductants in the surrounding has been proved to be of extreme importance. In this regard, the case of iron(II) can serve as demonstration of appropriate reductant efficiency [58]. In some yeasts and various fungi, though not *Saccharomyces cerevisiae*, special metal-siderophore interaction occurs (*Rhodotorula* – rhodotorulic acid siderophores) [59–61], which has the function

to promote bioaccumulation of particular ions. Originally, siderophores are chelating agents excreted by many microorganisms to facilitate the uptake of ferric ions [61]. However, there is some evidence that chromium(III) can also be inserted into siderophores. Resulting Cr(III)-siderophore complexes are kinetically inert (i.e. ligand exchange is slow) [60].

The trivalent state of chromium is thermodynamically the most stable and is commonly found in the living systems. Trivalent chromium has a strong tendency to form co-ordination compounds, complexes and chelates. Its co-ordination number is 6, and it can bind ligands to form hexaco-ordinate or octahedral complexes. Consequently, this ionic complex has positive charge what makes it less suitable for transport into the cell. Free chromic ions do not exist in aqueous solutions. They are always co-ordinated, either with water or with other ligands in the solution. Metal-ligand bonds may involve oxygen, nitrogen or sulfur [12, 14, 44, 57, 62].

At neutral or basic pH, Cr-H<sub>2</sub>O bonds are modified and Cr-OH<sup>-</sup> bonds are created, leading to the formation of giant macromolecules (polymerization of hydrated Cr – known as olation) that precipitate and are thus biologically inert. Oxalate ions and other strong ligands can prevent and reverse olation, while weaker ligands like pyrophosphate, methionine, serine, glycine, leucine, lysine and proline prevent the reaction at physiological pH, what enables functioning of chromium in cell metabolism [12, 14, 57].

Following the message of Mertz [12], the most important oxidation state of chromium in biological systems is 3+ and is therefore not likely to participate in oxidation/reduction reactions. Furthermore, chromium(III) occurs mostly in octahedral configuration with coordination number 6 and its relative inertness of the chromium-ligand bond makes it suitable for structural function, like the stabilization of tertiary structures in proteins or nucleic acids.

The yeast tolerance to metal ions is key parameter in the conditions when yeasts are grown in media loaded with heavy metals. The variation in tolerance or resistance among yeast species can be the result of different cell wall compositions [35]. There is neither much data about the microbial requirements for chromium nor available data for chromium biosorption and bioaccumulation. Frequently it is regarded as a toxic element to which some microorganisms have evolved resistance mechanisms [64].

Most common sites of chromium binding in yeasts are in the cell walls and cell membrane structure. In yeast cell the compounds such as pyrophosphate, polysaccharides, proteins, amino acids (Table I) are potential donors of ligand donor groups [12, 27, 35, 38, 65]. They act as ligand donor groups through oxygen, nitrogen and sulfur [66].

**Table I***Amino acids with metal-binding groups in protein [adapted from 38, 60, 63]*

Amino acid	Side chain	Character	Metals-binding
Alanine	-CH <sub>3</sub>	Neutral	/
Arginine	-CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> NHC(NH <sub>2</sub> <sup>+</sup> )(NH <sub>2</sub> )	Basic	*
Asparagine	-CH <sub>2</sub> CONH <sub>2</sub>	Neutral	Ca
Aspartic acid	-CH <sub>2</sub> COOH	Acidic	Ca, Zn, Fe, M(II), M(III)
Cysteine	-CH <sub>2</sub> SH	Neutral	Zn, Cu, Fe, Ni, Mo
Glutamic acid	-CH <sub>2</sub> CH <sub>2</sub> COOH	Acidic	Ca, Zn, Fe, M(II)
Glutamine	-CH <sub>2</sub> CH <sub>2</sub> CONH <sub>2</sub>	Neutral	Fe
Glycine	-H	Nonpolar	Cr
		Hydrophobic	
Histidine	-CH <sub>2</sub> C <sub>3</sub> H <sub>3</sub> N(NH <sup>+</sup> )	Neutral	Zn, Cu, Mn, Fe, Ni
		Basic	*
Isoleucine	-CH(CH <sub>3</sub> )CH <sub>2</sub> CH <sub>3</sub>	Nonpolar	/
		Hydrophobic	
Leucine	-CH <sub>2</sub> CH(CH <sub>3</sub> )(CH <sub>3</sub> )	Nonpolar	Cr
		Hydrophobic	
Lysine	-CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> NH <sub>3</sub> <sup>+</sup>	Basic	*, Cr
Methionine	-CH <sub>2</sub> CH <sub>2</sub> SCH <sub>3</sub>	Nonpolar	Cu, Fe, Cr
		Hydrophobic	
Phenylalanine	-CH <sub>2</sub> C <sub>6</sub> H <sub>6</sub>	Nonpolar	/
		Hydrophobic	
Proline	-CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> NH <sub>2</sub> <sup>+</sup>	Nonpolar	Cr
		Hydrophobic	
Serine	-CH <sub>2</sub> OH	Neutral	Ca, Cr, M(I)?
Threonine	-CH(OH)CH <sub>3</sub>	Neutral	Ca, M(I)?
Tryptophan	-CH <sub>2</sub> C=CH(NH)C <sub>6</sub> H <sub>4</sub>	Nonpolar	/
		Hydrophobic	
Tyrosine	-CH <sub>2</sub> C <sub>6</sub> H <sub>4</sub> OH	Neutral	Zn, Fe, Mn, M(III), Ca
Valine	-CH(CH <sub>3</sub> )(CH <sub>3</sub> )	Nonpolar	/
		Hydrophobic	
γ-carboxyglutamic acid	-CH <sub>2</sub> CH(COOH) <sub>2</sub>		Ca
β-hydroxyaspartic acid	-CH(COOH)(OH)		Ca

\* Anion binding

Additionally, it was found in higher organisms that chromium can act as a substitute for magnesium (Mg(II)) in protein (enzyme) sites [38]. It is well known that many ions can interfere with each other [34]. In the case of chromium the action of nickel (Ni(II)) can be compared with chromium action because their co-ordination chemistry tends to be the same, both prefer octahedral co-ordination, and aluminium

(Al(III)) which, like chromium, has a slow rate of ligand exchange and molybdenum (Mo(III)) with a range of octahedral complexes, which resemble compounds of Cr(III) [38].

Regarding chromium as an ion of choice for yeast metabolism, from the mentioned information one could expect that there might be some important activities still to be elucidated.

### Chromium accumulation in yeast biomass

Already in 1978 it was noticed that the yeast *S. carlbergensis* was able to uptake chromium from the medium with ( $^{51}\text{Cr}$ -labelled) chromium chloride. The 5% (w/v) glucose supplement increased the uptake of chromium into the yeast cell. Further glucose feeding to the growth media during cultivation did not improve chromium chloride uptake [67]. Their comments that diffusion is the only transport system into the cell have not been discussed since then. They based their conclusion on repeating glucose feeding. Much later it was reported that chromium(III) has to be organically bounded to be able to pass through the cell membrane [32]. Final explanation is still needed. For chromium(VI) it is claimed to use sulfate and phosphate transport route. Sulfate transporter has a function to translocate chromium(VI) as chromate ( $\text{CrO}_4^{2-}$ ) through the membrane into the cell because sulfate space conformation is very similar to chromate [68, 69]. Since first isolation of cDNA encoding High affinity sulfate transporter [70] there were published also other genes involved in transporting sulfate as well as chromate [71].

The complex structure of yeast envelope [37] protects vital processes in the cell. Chromium ion has to pass different barriers, the cell wall and membrane from the environment to the cell interior [72]. In the translocation process of metal ions into the yeast cell values for pH, temperature, metal's biological availability, etc. should be considered as the most important environmental parameters. When the intracellular metal ion concentration rises to the critical level, it can interfere with vital processes resulting in cell death in the mechanism of metal ion translocation [2, 73].

The metal ions uptake is essentially a biphasic process consisting of a metabolism-independent and a metabolism-dependent step. The initial biosorption step for metal ions is rapid [74, 75], typically only a few minutes in duration [76], and is independent of temperature [77]. In the case of chromium it was found a correlation in yeast *S. cerevisiae* between pH and chromium(III) uptake at different starting pH values. The studied pH range from 2 to 6 showed chromium uptake up to 1.54  $\mu\text{mol/g}$  dry biomass which was conducted at 28 °C for 4 hours in gently mixing conditions

(200 rpm) [78]. Under the applied experimental conditions the cell surface deposition capacity for chromium (CSDC-Cr) of the yeast cells increased with the pH. The initial binding step is thought to involve the microbial cell surface structure (e.g. cell wall components – polysaccharides). This agreed with the observation for other metal ions in the same pH range [79]. Complex growth media reduce bioavailability of the chromium(III) ion for yeast metabolism. pH value around 6 additionally enhances the problem of olation mentioned earlier. To avoid the problem of chromium(III) olation in complex aqueous media the pH 4 was found to be suitable for yeast cultivation [80].

*Fig. 1. Impact of chromium supplementation (5 mM Cr(III)) in two different phases of bioprocess (at the beginning and in the exponential phase) on its uptake in aerobic cultivation of Candida intermedia ZIM 156 (82)*



In the absence of the metabolic activity non-viable biomass can still sequester metal ions from surroundings by biosorption [74, 77]. Chromium biosorption capacity of non-viable yeast biomass (heat inactivated) compared to viable counterpart from the same source was 2.5-fold higher [81] what is in favour of technological application in environmental technology (i.e. removing chromium from water streams) when low concentration of metal ion is to be eliminated.

When metal/biomass ratio was below 0.1  $\mu\text{mol/g}$  dry mass Brady and Duncan [75] claimed that metal ion uptake to biomass was dependent on biosorption. *S. cerevisiae*, when glucose was added to the system where biomass and chromium were in the suspension, reached the ratio 0.577  $\mu\text{mol}$  of chromium per g dry yeast biomass, what indicates synchronous biosorption and bioaccumulation. Consequently, in viable yeast biomass chromium uptake was enhanced up to 5-fold [80, 81].

Studying chromium uptake in yeast, biosorption is not of primary importance over bioaccumulation, because chromium enriched biomass by cultivation was found more suitable for food and feed consumption.

If one takes into account mechanism for metal bioaccumulation [9], then active transport indicated is in response to electrochemical proton gradients, generated by membrane-bound  $\text{H}^+$ -ATPases, across the cytoplasmic and vacuolar membranes. Experimental results suggested the involvement of metabolic activity in chromium(III) ion bioaccumulation. Namely, concentrations of chromium ions higher than 2.40 mM stimulated a more intensive extrusion of  $\text{H}^+$  ions from yeasts and glucose even enhanced the proton export out of the cell [80]. Additionally, it was found that some heavy metals can release other cations from the cell (i.e. K(I), Mg(II)). This was observed by exposure of yeast cells to copper [79] but is no data for chromium available.

However, there is no other information whether known metal uptake systems have the capacity to transport chromium(III) ions or whether specific chromium ion transport into the cell even exists. Appropriate timing for chromium supplementation into growth media at different yeast growth phases was proved to be important for chromium bioaccumulation. The results showed that yeasts growth at higher chromium concentration added (10 mM) at the beginning of the bioprocess was more inhibited but chromium(III) uptake into yeast biomass was higher.

Addition of chromium in the middle of the exponential growth phase had no notably effect on yeasts' growth but resulted in approximately 30% lower chromium uptake. Cultivation of yeast *C. intermedia* ZIM 156 in 10 mM chromium(III) doubled (2400  $\mu\text{g/g}$  dry mass) its uptake in comparison to 5 mM concentration in the medium [82]. Similar experiment with *C. utilis* showed growth inhibition at lower concentration [50].

*Fig. 2.* Concentration of chromium in biomass of *Candida intermedia* ZIM 156 in batch and combined batch/fed-batch culture in medium supplemented with chromium(III) ( $100 \mu\text{M KCr}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ ) after 20 hours cultivation (Total chromium (A) and organically bounded chromium (B)) [51]

Yeast growth and chromium bioaccumulation in biomass was studied in batch, fed-batch and continuous culture. Regarding chromium effects on biomass production, the batch cultivation mode was found to be better than combined batch/fed-batch. According to Kosman [83] this can be attributed to biosorption capacity of yeast biomass and better uptake of chromium in the beginning of the exponential growth

phase. Such behaviour favors batch over combined batch/fed-batch operating mode in bioaccumulation of chromium(III) [78].

Consequently, total chromium accumulation in batch cultivation was 4.5-fold higher compared to combined batch/fed-batch cultivation mode with yeast *C. intermedia* ZIM 156 [78]. On the contrary, combined batch/fed-batch cultivation mode was found to be beneficial over batch biomass production because of high concentration of organically bounded chromium in yeast biomass and technologically suitable specific growth rate ( $\mu_{\max}=0.26 \text{ h}^{-1}$ ).

The high efficiency of this bioconversion can be seen through total vs. organically bounded chromium ratio in yeast biomass of *C. intermedia* ZIM 156 (Fig. 2) [51]. The organically bounded chromium is considered to be the fraction, which can be extracted from total yeast biomass with  $\text{NH}_4\text{OH}$  [84].

When combined batch/fed-batch cultivation mode is analyzed, yeast *C. intermedia* ZIM 156 was found to be capable of chromium bioconversion into organically bounded chromium fraction as high as 60%. Combined batch/fed-batch cultivation mode showed a 9-fold better chromium ratio in spite of a 22% lower total chromium concentration in yeast biomass.

On the other hand *C. intermedia* ZIM 156 cultivated in batch culture was able to bioconvert 12%, when compared to yeast *S. cerevisiae* ZIM 198 cultivated in the same conditions which was able to convert 23% of chromium which came into the cell into organically bounded fraction. The fraction of organically bounded chromium (org. Cr/tot. Cr) remained constant (6%) in yeast biomass when supplementation with chromium(III) cross 1.44 mM. In bioprocess with 0.96 mM chromium(III) *Saccharomyces cerevisiae* was metabolically capable to incorporate 3.9-fold higher portion of chromium into organically bounded fraction what had a consequence in chromium concentration factor (CCF) in yeast biomass which attained 236-fold higher value than in yeast biomass from control media (without chromium(III) supplementation).

The highest intracellular chromium binding capacity was found in *S. cerevisiae* for organic fractions in the molecular weight range from 10,000 to 100,000 and more [78, 80]. Under normal conditions (no addition of chromium to growth media) chromium distribution was observed in a range of molecular weights between 10,000 and 3000 (Fig. 3). Many cytoplasmic biomolecules have the ability to bind metal ions. The diversity of intracellular organelles provides a wide range of potential binding sites [38, 63, 85].

Fig. 3. Chromium organically bounded incorporation factor of different molecular weight fractions of yeast *S. cerevisiae* grown in continuous culture with 28  $\mu\text{M}$  of glucose at dilution rate  $0.08 \text{ h}^{-1}$  in medium loaded with 96  $\mu\text{M}$  of chromium [80]

Regarding chasing chromium distribution among biomolecules similar results were obtained in biomass of yeast *C. intermedia* isolated as intracellular low-molecular-weight chromium-binding biomolecules [86]. Determination of chromium in cell fractions of biomass collected during cultivation in chromium enriched medium revealed that it was present in all collected chromatographic peaks, however, chromium was unevenly distributed. The comparison of low molecular weight fractions in chromium enriched biomass with control sample showed remarkable difference in chromium distribution and its loading. Fractions with highest chromium concentrations were found in molecular weight range from 1500 to 7000. Two distinguishable peaks in this range contained the highest amount of chromium [86].

Binding sites of chromium, compartmentalization, localization in the cell and finally incorporation processes into yeast biomass are rather complex processes and many details remain to be elucidated. On practical scale we gained some experience to push yeast to accumulate chromium. There are many patents claiming efficiency of

their bioprocessing strategy for yeast biomass enrichment with chromium [87–91] and just few publications [49, 50, 92], what confirm strong economical interest for this product in the future. This is not unusual since yeast biomass enriched with chromium is a source of GTF molecule (glucose tolerance factor) [42, 52, 93–96]. GTF molecule is actually a Cr-binding molecule, which influences glucose metabolism in humans. Some authors claim that GTF activity is correlated with the amount of chromium accumulated in yeast [97] but so far this observation is not clearly proved. Moreover, GTF function in yeast metabolism seems to be important, since there are experiments showing that GTF enhances glucose consumption also in yeast metabolism when added to the growth medium [98, 99].

However, its structure remains to be discovered in spite of many attempts in the past [54, 100–106]. The challenge of GTF is still not completely elucidated with its function in glucose and lipid metabolism [107, 108], in particularly to actual active substance in its molecule [54, 100–104] and with regards to its mechanism [106, 109–111].

Due to the importance of GTF product there have been some attempts to make GTF biosubstitutes as already mentioned [112].

**Table II**

*Chromium concentration in yeast biomass [113, 114]*

Yeast	Chromium concentration ( $\mu\text{g/g}$ dry mass)
<i>S. cerevisiae</i>	0.7–4.4
<i>C. lipolytica</i>	1.6–3.0
<i>C. boidinii</i>	1.1–5.1
<i>H. platypodis</i>	3.2
<i>P. kluyveri</i>	3.0
<i>Y. lipolytica</i>	0.22
<i>S. starkeyi-henricii</i>	0.19

The natural source of chromium (i.e. enriched yeast biomass) was proved to be more beneficial to glucose metabolism than Cr-picolinate [115, 116] or other Cr-compounds [117–120]. Even more, some publications claim that Cr-picolinate can be cancerogenic [121, 122], what is not the case with chromium enriched yeast biomass [123]. Following current results in the literature and our own results it is obvious that the majority of research is focussed on yeast in aerobic conditions. Anaerobic

environment, in spite that it is widely extended in technology, is not comprehensively approached in the past. The presence of chromium in yeast biomass originated from anaerobic bioprocesses was also studied in traditional process of must fermentation and it was found that chromium had beneficial effect in *S. cerevisiae*. However, stronger production of the reduction compounds were found, consequently [124].

### **Effect of chromium on yeasts' viability**

Chromium can have different effects on yeast metabolism. It can be beneficial if the concentrations are in physiological range. Deficiency symptom from the lack of chromium is not known for yeast, but toxic effects from the excess of it can be recognized clearly with growth inhibition or with analyzing particular biomolecules in the cell (i.e. DNA, RNA, proteins).

Since chromium and also some other metal ions are both essential and potentially toxic, their intracellular concentrations are the subject of precise homeostatic regulation [6].

As chromium is important for humans there were some attempts in the past to isolate yeast with high tolerance to it [125–129].

The problem of metal ions' toxicity and/or resistance is discussed in the literature [19, 57].

The adequate methodology for microbial bioassays for the detection of metal toxicity is important [130–132], since different approaches can give different results, which are not comparable at all. If one wants to optimize growth medium, the tolerance to particular metal ion for selected *yeast* species has to be determined [64]. The survival of living cells depends on their ability to react to alterations in the environment and to appropriately respond to the newly emerged conditions [133]. Knowledge of stress and stress responses is crucial to understand how single-cell and multicellular organisms adapt to changing environmental and physiological conditions [134, 135]. The molecular mechanisms induced upon the exposure of cells to such adverse conditions are commonly designated as stress responses. The aim of stress response mechanisms is to protect cells against the potentially detrimental effects of stress challenges and to repair any molecular damage, and to adjust the metabolism and other cellular processes to the new status [133]. These stress responses involve aspects of sensing, signal transduction, transcriptional and post-translational control, protein-targeting to organelles, bioaccumulation of protectants, and activity of repair functions [136].

Redox metals play a major part in the generation of reactive intermediates (free radicals, other reactive intermediates) in the cell [19, 133] which are potentially the most dangerous for the cell.

To avoid the problems of stress and consequently toxicity of ions in cell surroundings it is crucial to know yeast tolerance limit and spectrum of ions dangerous for the cell. In case of chromium the screening procedure on agar plates based on concentration gradients was developed (fast and reliable), which can serve for rapid assessment of yeast tolerance to potentially toxic metal ions [128, 137, 138].

These screening tests showed great inter- and intra-generic differences among 23 yeast genera and 49 species. Yeasts from genera *Saccharomyces*, *Candida*, *Hansenula* and *Kluyveromyces* represented more than 50% of strains tested. The tolerance to chromium(III) was found to be normally distributed and 6% of screened population could tolerate up to 6.9 mM Cr(III) concentration in the medium. Majority (approx. 40%) of yeast strains tolerated up to 5.8 mM and just 4% showed tolerance below 1.5 mM Cr(III) [138].

The mathematical model was established with an aim to characterize yeast viability and mortality.

On the other hand, if more precise data are needed, an advanced procedure may be introduced for precise measurement of yeast viability or mortality. It is more time consuming, but it gives more concise information on yeast characteristics [140]. Viability was defined with four parameters: population condition, cell viability in the buffer and cell viability in the buffer supplemented with the effector and specific cell viability. Mortality was defined with three parameters: cell mortality in the buffer, cell mortality in the buffer supplemented with the effector and specific cell mortality. Parameters were calculated on the bases of total cell count per milliliter and number of viable cells per milliliter in each experimental step.

This model was tested for five chromium(VI) compounds on seven selected yeasts (Fig. 5). The study elucidated that physiological status of yeast population from particular species is not comparable in spite of equal treatment during cultivation.

Population condition demonstrated this status with values in the range from 2.78 to 48.58% in theoretical scale of 100%. With this model one is able to draw conclusion of chromium to yeast viability/mortality as can be seen in Figure 4.

Six chromium(III) compounds were assessed by the same model (Fig. 6). Studies of chromium(III) compounds revealed deviations, which could be attributed to different chemistry of chromium(III) and chromium(VI) compounds in aqueous solution [73, 140].

*Fig. 4. Model for graphical determination of specific cell viability and specific cell mortality at different concentration of Cr(VI) for yeasts Saccharomyces cerevisiae ZIM 321 and Schwanniomyces occidentalis ZIM 763 [140]*

The aim of these experiments was also to compare toxicity of chromium(III) to chromium(VI) compounds, which are known to be more toxic. The results showed that different species of yeast can exhibit noticeable differences in their ability to tolerate higher concentrations of chromium(III) and chromium(VI).

$\text{CrCl}_3 \times 6\text{H}_2\text{O}$  and  $\text{Cr}(\text{NO}_3)_3 \times 9\text{H}_2\text{O}$  exhibited the highest toxicity in the case of *Saccharomyces cerevisiae* ZIM 753 and *Candida intermedia* ZIM 156, while very low concentrations of all Cr(III) compounds except  $\text{KCr}(\text{C}_2\text{O}_4)_2 \times 3\text{H}_2\text{O}$  were toxic to *Saccharomyces diastaticus* ZIM 152. However, all tested yeasts showed the greatest survival in the case of  $\text{KCr}(\text{C}_2\text{O}_4)_2 \times 3\text{H}_2\text{O}$  [141].

We found *S. cerevisiae*, *S. paradoxus* and *C. intermedia* to be the most tolerant where an average specific cell viability for all Cr(VI) compounds at the highest Cr(VI) concentration of 1000  $\mu\text{M}$  was in the range from 13–25% and 0–2% in the case of less tolerant yeasts *S. bayanus*, *S. pastorianus*, *S. diastaticus* and *S. occidentalis*. On average the most toxic compound for all yeasts at all Cr(VI) conc. was  $\text{KCrClO}_3$  and the least toxic compound was  $\text{K}_2\text{CrO}_4$  [139, 141].



*Fig. 5.* Yeast population viability calculated from the data for yeasts included into the study (*S. cerevisiae*, *S. paradoxus*, *C. intermedia*, *S. bayanus*, *S. pastorianus*, *S. diastaticus*, *S. occidentalis*) presented for particular Cr(VI) compounds [139]

*Fig. 6.* Effect of higher concentrations of Cr(III) compounds: 1 =  $\text{CrCl}_3 \times 6\text{H}_2\text{O}$ , 2 =  $\text{Cr}(\text{NO}_3)_3 \times 9\text{H}_2\text{O}$ , 3 =  $\text{CrK}(\text{SO}_4)_2 \times 12\text{H}_2\text{O}$ , 4 =  $\text{KCr}(\text{C}_2\text{O}_4)_2 \times 3\text{H}_2\text{O}$ , 5 =  $\text{Cr}(\text{CH}_3\text{COO})_3$ , 6 = Cr-citrate on specific survival of yeast *Candida intermedia* ZIM 156 [141]

This might be related to the involved mechanism of detoxification [3, 142], which is based on intracellular quarantine in particular compartment or on blocking transport into the cell.

The most common essential amino acid used in the detoxification of many metals is cysteine, which is found in some yeasts as a dominant residue in the thioneins, or as a component of the  $\gamma$ -glutamyl peptides which may bind metals. Another possibility is that these proteins might be involved in pumping out of the cell. However, there are some indications in this respect for some metabolically important metals like Zn(II) [35], but is no data for chromium so far.

Detoxification mechanisms are studied by some researchers lately and they showed that vacuole is involved in this action in normal metabolism in the cell detoxification. When vacuolar-lacking mutants of *Saccharomyces cerevisiae* were exposed to chromate, they displayed higher sensitivity. However, chromium is bioaccumulated into the cytosolic compartments and not to vacuole also in wild strains [143].

One approach for studying toxicity and tolerance of yeast to chromium(VI) or chromium(III) can be established analytically with a goal to determine intracellular components, which turn on or disappear during prolonged exposure to chromium. During the cultivation of *S. cerevisiae* at dilution rate (D)  $0.08 \text{ h}^{-1}$  chromium ions exert a harmful effect on yeast cell growth, cell protein and RNA concentrations [144], what was also the case in batch growth [145]. The toxic effect of chromium ions was found as 21% reduction of biomass, 9% reduction of protein and 22% reduction of total cell RNA during continuous cultivation. In batch cultivation, the effect of chromium ions was recognized in growth rate reduction and extension of lag phase [80].

Analyzing distribution of chromium in the cell showed that fractions with molecular weight higher than 100,000 support the mechanism of detoxification at high concentration levels of intracellular chromium in *S. cerevisiae*. Determination of chromium in this cytosolic biomolecules with high molecular weights supports the idea that fast binding of toxic intracellular chromium ion is possible. This is the generally accepted mechanism of action of other heavy metals [146].

Among cell biosynthetic products, metallothionein, the specially designed proteinous molecules for attracting metals (especially toxic ions) are discovered. One group of metallothionein-like molecules found in yeast *S. cerevisiae* is the most studied low molecular weight-cysteine-rich protein for Cu binding [147]. Lately, Ballatori claimed glutathione mercaptides as transport forms of metal ions in the yeast cells [148].

### Effect of chromium on yeast genome

Toxicity of chromium is discussed elsewhere [55, 149, 150] since it is important in industry and in life processes. However, interference and all positive and/or negative actions of chromium compounds within the yeast cell and its metabolism is focussed on genome since cumulative mutation can effect future development and expression of genetic informations in DNA structure.

According to early observation, chromium (VI) is areactive towards DNA under physiological conditions *in vitro* [69]. However, *in vivo* the reduction of chromium(VI) by cellular reductants in cell culture (the microsomal cytochrome P-450 system, mitochondrial electron transport chain complexes, aldehyde oxidase, hydrogen peroxide, scorbate and thiols) to the reactive intermediates, including Cr(VI) thioester, Cr(V), Cr(IV) species and free radicals (hydroxyl and thiol radicals), is thought to be an important step in the mechanism of Cr(VI) induced DNA damage [69, 151–155].

There are many publications dealing with DNA lesions induced by chromium(VI) *in vivo* as well as chromium(III) compounds *in vitro*, but they seldom use yeast as a model organism. One can find publications on Cr-DNA adducts, DNA-DNA cross links (DDC) [156, 157], DNA-proteins cross links (DPC) [158] and DNA brakes- single and double [68, 56, 154, 159–161], proving direct reactivity of chromium with DNA. Some of them are dealing with an interference of chromium(III) compounds *in vitro* with enzymes in DNA processing (i.e. polymerase) [152, 162], or with chromium bounded to DNA on the way to alter cleavage side for restriction endonucleases [163]. Many publications deal with free radicals in gene toxicity. Combination of hydrogen peroxide and chromium(III) and chromium(VI) showed DNA damage mediated with Fenton-like reaction [164–166]. Further interesting observation that metal ion-dependent hydrogen peroxide-induced DNA damage is more sequence specific than metal specific opened new dimension of the problem [167].

Few publications claim interference with particular system included in detoxification in the cell. Alcedo and co-workers found chromium(VI) altering metal-inducible expression, but not basal expression of the metallothionein gene. They used this as a proof for suggesting that chromium(VI) has priority to inducible genes [168].

Regarding mutagenesis in yeast there were many publications dealing with the problem of chromium twenty years ago. The majority of them were oriented just to detect mutagenic effect of chromium in different preparations [117, 120]. However, there are some publications, which gave suitable start for further research in the field, like induction of petit mutants by calcium chromate. Its toxic and mutagenic effects on the mitochondria of *S. cerevisiae* were showed [169, 170]. In the same organism,

induction of reverse mutation and mitotic gene conversion by some metal compounds was demonstrated [171].

In the last decade, rapid estimation of chromosomal damage in yeast (i.e. radiation-induced) has been evaluated by pulsed field gel electrophoresis [172–174]. The effects of chromium(VI) compound  $K_2Cr_2O_7$  can be visualized in the same manner [175]. PFGE together with densitometry offers opportunity to follow DSB (Double-Stranded Brakes), which were detected as smear of DNA on the gel after yeasts were exposed to chromium(VI). They found that in *in vivo* conditions chromium(VI) compound  $K_2Cr_2O_7$  induced double-stranded brakes of chromosomal DNA in all *S. cerevisiae* tested.

Further studies revealed that recombination repair (gene Rad51) reduced gene toxicity of chromium compounds in exponential growth phase for all applied concentrations up to 20 mM of chromium compounds. Excision repair of DNA damage (gene RAD1) was found in the stationary growth phase as crucial for reducing gene toxicity of  $K_2Cr_2O_7$ .

*Fig. 7. The effects of chromium(III) compound  $K_2Cr_2O_7$  on chromosomal DNA in yeast *S. cerevisiae* ZIM 465 *in vitro* (S1-detected through S1 nuclease activity-cutting SSB on DNA; OD–Optical density) [176]*

Additionally, the cells were exposed to chromium(VI) in exponential growth phase and they evidently have excision repair mechanism at high concentration range.

Since lethality is correlated to chromium concentration, the study of cell response showed gene RAD18 responsible for mutation repair. This gene is active in exponential growth phase, but not in stationary phase [176]. As a consequence of  $K_2Cr_2O_7$  treatment, chromosomal aberrations were found in *S. cerevisiae in vivo*, specifically on one chromosome (Figure 7). Proposed chromium induced damage was confirmed by survival test with rad51 mutant, since rad51 mutant has no mechanism of repair as is common in wild strain, consequently their survival was strongly reduced. Single-stranded breaks (SSB) were tested in *in vitro* conditions with chromium(III). Nuclease S1 showed to be appropriate tool for detection of spots at chromosomal DNA in *S. cerevisiae* where this DNA damage could occurred. Results suggested that chromium(III) induces structural changes in DNA structure and consequently SSB [176]. Today it is stated that chromium-induced DNA damage can be repaired after mutagenesis [177, 178], since repair mechanism are not damaged by chromium oxidation.

Cells have evolved a number of capabilities to repair macromolecules that are damaged and to inactivate reactive intermediates. Yeast cells contain enzymatic as well as non-enzymatic defense mechanisms against the harmful effects of reactive deviates. The genes encoding components of this defense apparatus, such as those involved in glutathione or thioredoxin biosynthesis, display increased expression after stress challenge. Glutathione and thioredoxin are normally involved in cellular redox-reactions, in particular those involved in the establishment and maintenance of the tertiary structure of proteins [133].

### Conclusions

Chromium is an element in yeast metabolism, but when accumulated in excess, becomes toxic. The form and concentration of chromium in the macro- and microenvironment of the yeasts are important. Chromium(VI) with its negative charge enters cell more easily than chromium(III), which is positively charged in physiological conditions. However, all the transport mechanisms are still not clearly defined. Since toxicity is connected with chromium concentration, its manifestation in metabolism is proportional with amount, which can enter the cell and interfere with biological functions. DNA was showed to be damaged by chromium(VI) *in vivo* and chromium(III) *in vitro*. Single-stranded and double-stranded breaks are induced by chromium. During cultivation in batch, fed-batch and continuous processes reduction of RNA, proteins and biomass yield are present. However, there are yeasts, which tolerate higher concentrations of chromium and are more suitable for bioprocessing.

Their fast screening can be conducted by the gradient agar plate method, followed by cell viability/ mortality method, which select adequate yeast. Yeast biomass showed to be able to uptake high amount of chromium by biosorption and bioaccumulation. The ratio between total and organically bound chromium was good. However, the function of chromium in glucose tolerance factor and role of chromium in yeast metabolism remains in focus of current research. The question if metallothionein-like complex, which is able to bind chromium in yeast remains to be opened for further research.

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