Chromium uptake by *Saccharomyces cerevisiae* and isolation of glucose tolerance factor from yeast biomass

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Fermentations with yeast *Saccharomyces cerevisiae* in semiaerobic and in static conditions with the addition of chromic chloride into the used molasses medium were analysed. It was proved that the addition of optimal amounts of $CrCl_3$ into the basal medium enhanced the kinetics of alcohol fermentations. The addition of 200 mg/l $CrCl_3$ into the medium stimulated both the yeast growth and the ethanol production in all experimental conditions. On the other hand, the results showed that Cr^{3+} ions were incorporated into yeast cells during fermentation. Under these conditions the accumulation of Cr^{3+} ions was performed by yeast cells during the exponential growth phase, and with enriched amounts of 30–45 $\mu g/g_{d.m.}$ of cells.

Yeast biomass enriched with chromium ions was extracted with 0·1 mol/l NH_4OH assuming that the extracts had the glucose tolerance factor (GTF). Then the extracts were passed through a gel-filtration column in order to isolate and purify the GTF. The presence of GTF in the purified fractions was determined by measuring the absorbance at 260 nm.

It is evident from the obtained results that the added purified fractions enhanced the rates of CO_2 production as well as the glucose utilization during alcoholic fermentation. As expected, the enhancement of both rates depended on the amounts of extracts added to the fermentation substrate. Thus, it is evident that purified extracts contained the GTF compound, and that Cr^{3+} ions were bonded to the protein molecule.

1. Introduction

The role of microelements in the metabolism of higher organisms and yeasts has recently become a very interesting field of research work (Jones 1990). Particular attention has been given to the effect of zinc, copper, iron, chromium, selenium, manganese and other trace elements in the prevention of certain diseases in humans and animals as well as their role in the yeasts metabolism (Jones 1990; Mertz 1993). It is also known that some microelements (Zn, Cu, Cr, Fe) may influence the stability of cell membranes, as well as the syntheses of nucleic acids and the stability of the double helix of DNA while forming hydrogen bonds (Beran *et al* 1995; Davis and Vincent 1997).

Metal ions can be intracellulary accumulated by fungi even from low external concentrations. Yeasts have several properties which can lead to an increased resistence to toxic metals (White and Gadd 1987). Two important mechanisms may be (i) reduction of metal uptake or impermeability and (ii) sequestration of metals in and/or around the cell.

Reduced metal uptake has been noticed in several metal-tolerant strains of fungi and yeasts. Sequestration can occur as an extracellular precipitate, for example, a sulphide, partitioning among cell compartments or binding to the specific intracellular molecules such as metalothioneins and polyphosphates (White and Gadd 1986).

Keywords. Glucose tolerance factor; *Saccharomyces cerevisiae*; yeast enriched with chromium

During alcohol fermentation, the addition of micro-elements into yeast cells is of particular interest. The role of chromium in yeast has still not been completely clarified. On the other hand, its toxicity is often observed during the yeast growth and alcohol fermentation. The importance of chromium as a nutrient factor was established for the first time when it was found out that brewer's yeast could positively influence the carbohydrate metabolisms of higher organisms enhancing the insuline activity (Demirci and Pometto 2000).

It has been established that chromium, present even in the trivalent state, does not readily convert into its biologically most active form in mammals and micro-organism (Kompulainen *et al* 1978). Trivalent chromium has a very strong tendency to form octahedral complexes with biological ligands on the cell's membrane (Kompulainen *et al* 1978). Mannan-*b*-glucan is the main structural polymer of the cell wall in *Saccharomyces cerevisiae*. Other components include proteins, lipids and pigments and this diversity is reflected by the presence of a range of distinct, potential metal-complexing sites, e.g. carboxylate, phosphate, sulphydryl and amine groups (Rapoport and Muter 1995).

The particular role of trivalent chromium in the maintenance of normal carbohydrate metabolism in mammals and yeasts has since been extensively reviewed (Mertz 1993). In addition to the carbohydrate metabolism, chromium may have other physiological functions (Kompulainen *et al* 1978). Literature data suggest chromium as an element which can stabilize the tertiary structure of proteins and the conformation of the cell RNA and DNA (Demirci and Pometto 2000).

Chromium deficiency in humans results in symptoms comparable to those associated with diabetes. Systems that specifically respond to chromium are, for example, the activation of insulin, which is the regulating hormone of the blood glucose level (Mertz 1993; Toepfer *et al* 1977). The role of Cr^{3+} ions in the metabolism of mammals is connected with the glucose tolerance factor (GTF). This is a cationic Cr^{3+} complex of low molecular weight. Detection of its purified fraction has revealed that this complex factor consists of Cr^{3+}, nicotinic acid, and the amino acids glycine, glutaminic acid and cysteine (Toepfer *et al* 1977). This synthetic complex of Cr^{3+} and these ligands is biologically active in a similar way as GTF isolated from yeast (Toepfer *et al* 1977; Beran *et al* 1995). It is also believed that its biological role in yeast cells is mainly connected with carbohydrate metabolisms (Ducros 1992).

GTF has been isolated from brewer's yeast as the most active form of Cr. Its production from the yeast cells has been of particular interest (Davis and Vincent 1997). Yeast cells enriched with Cr^{3+} ions can serve as a pharmaceutical preparation. These products have already reached the world market (Beran *et al* 1995).

However, in most publications, the isolation of GTF was described as taken from the yeast cells previously enriched with chromium. Partially purified extracts enriched with this factor were used in tests on diabetic mice. It was established that the preparation of this factor had a favourable effect on the reduction of the glucose level in blood of diabetic mice (Davis and Vincent 1997).

Therefore, the aim of the present study was to incorporate optimal concentrations of Cr ions into the cells of baker's yeast *S. cerevisiae* during cultivation in semiaerobic and static conditions with the addition of 200 mg/l $CrCl_3 \cdot 6H_2O$ into the basal medium because in the preliminary experiments (data not shown) that amounts of chromium ions were optimal for yeast growth. GTF was extracted and purified from the biomass enriched with Cr^{3+} ions. Purified GTF extracts were added to the synthetic medium to establish its impact on the alcohol fermentation and the growth rate of the yeast *S. cerevisiae.*

2. Materials and methods

The yeast used in this study was fresh baker's yeast *S. cerevisiae* with 30% of dry matter obtained from the "Kvasac, d.o.o.", Savski Marof-Croatia.

2.1 *Batch process*

The composition of the basal medium for yeast biomass cultivation was (g/l): beet molasses, 90 (corresponding to 50 g/l sucrose); $(NH_4)_2HPO_4$, 2; $MgSO_4$, 0·5. The pH of the medium was adjusted to 4·5 with H_2SO_4 (c = 0·5 mol/l). The medium was sterilized at 120°C for 10 min and, after cooling to 30°C, it was centrifuged at 2000 *g* for 10 min. The clear supernatant was used as the basal medium (BM). For batch processes 500 ml Erlenmeyer flasks with 200 ml of BM were used. Cultivations were performed in BM with and without the addition of 200 mg/l of chromic chloride in semiaerobic (shaker) and static (thermostat) conditions at 30°C for 10 h.

Samples were analysed three times for biomass (expressed as biomass dry matter) and ethanol concentrations parallely. The results represented the data worked out statistically through mean values and standard deviations and the differences between the effects were estimated by Students' *t*-distribution (Dixon 1957).

The harvested and washed yeast biomass was treated with 0·1 mol/l NH_4OH. The samples were shaken on a rotary shaker at 175°/min at 30°C during 1 h (Anderson *et al* 1978). The extracted samples were then passed through a column on Sephadex G-75 (Barseghian 1987).

The fractions collected on Redi Frac collector were measured for optical absorbance at 260 nm.

CO_2-gas production was measured after 4 h of fermentation (static conditions) in a synthetic medium. The initial concentration of yeast biomass was 0·75 $g_{d.m.}$/l (static conditions) with addition of 10 ml fractions collected from eluation procedure with highest absorbances. This amount of fractions was added because it was observed that it significantly increased CO_2 production (data not shown). The composition of the synthetic medium was (g/l): glucose, 20; bacto pepton, 10; yeast extract, 5.

The kinetics of glucose utilization rate was also monitored during 6 h of fermentation (static conditions) in the same medium (synthetic). The initial concentration of yeast biomass was 0·75 $g_{d.m.}$/l with addition of 10 ml fractions with highest absorbances.

2.2 *Analysis*

Dry matter of yeast biomass was determined by drying yeast biomass at 105°C to a constant weight after centrifuging 5 ml of samples at 2000 *g* for 10 min on a portable centrifuge (Tehtnica LC-320). The concentration of ethanol in the medium was determined by Martin-Dietrich method (Kretschmar 1955) and chromium ions concentration in yeast cells and extracts were analysed by using "Varian" Spectra AA 300 Atomic Absorption Spectrophotometer fitted with a 10 cm single slot burner head, and by using an air-acetylene flame. Chromium concentration was determined by reference to an appropriate standard metal solution (Varian Techtron 1989). Protein concentration was determined by Kjeltec-system (1976), type 1002.

Fractioning of the extracted samples was performed on a gel-filtration column with Sephadex (Beran and Stahl 1995). Collected fractions on Redi Frac Collector were detected spectrophotometrically at 260 nm wavelength. CO_2-gas production was measured by the Orsat method (Welcher 1963). D-glucose concentration in the medium was detected by the enzymatic kit of Boehringer Manheim spectrophotometrically at 365 nm wavelength.

3. Results and discussion

The yeast *S. cerevisiae* is a good source of biologically most active form of chromium. Biological material contains an organic chromium (III) complex whose chemical structure and biological activity have only partly been identified (Kompulainen *et al* 1978). Chromium as a part of GTF differs distinctly from synthetic chromium compounds in its biological activity. The similarity of the biological, chemical, and physical characteristic of natural and synthetic preparations strongly suggests, but does not prove, the niacin-chromium-niacin axis as the essential GTF configuration. The optimal composition of the amino acid ligands is still unknown, as is the functional group on the nicotinic acid to which chromium is coordinated (Toepfer *et al* 1977). In most models two nicotinate ligands are coordinated as monodentate ligands in trans-positions via oxygen or nitrogen donor atoms. However, both biological extracts and synthetic GTF models have not been properly characterized (Galuszka *et al* 1998). The availability for absorption and biological activity of at least one of these compounds is much greater than it is with inorganic chromium or organic chromium (III) complexes prepared synthetically (Galuszka *et al* 1998).

Mertz *et al* (1974) have established that brewers' yeast (*Saccharomyces carlsbergensis*) is the best source of this biologically most active form of chromium compounds.

Figures 1 and 2 show the kinetics of biomass and alcohol production with and without the addition of chromic chloride in the basal medium.

A dose of 200 mg/l chromic chloride was added into the basal medium because, in preliminary investigations, it had been observed that this quantity of $CrCl_3$ was optimal for enhancing the growth rate of yeast cells. A stimulative effect, with addition of $CrCl_3$ to the basal medium, could be explained by the fact that chromium accelerated the metabolism of carbohydrate, catalyzed the phosphoglucomutase reaction and stimulated other enzyme systems, for example the succinate-cytochrome reductase (Moore 1990).

With regard to the fact that the accumulation of chromium ions into yeast cells was completed in the exponential growth phase (unpublished data), the time

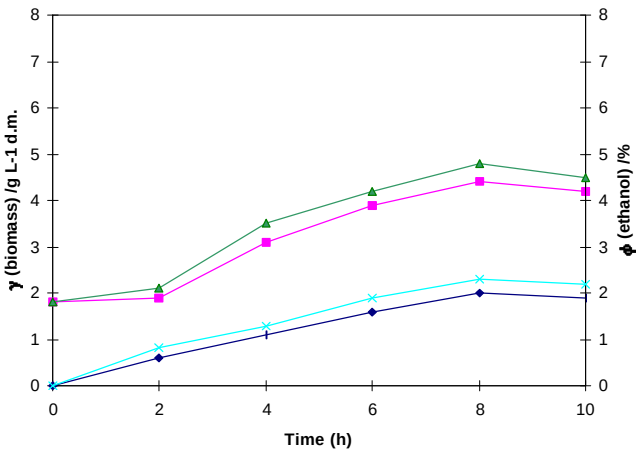

Figure 1. Dynamics of biomass and ethanol production in static conditions with 200 mg/l of $CrCl_3$ and without addition of $CrCl_3$ into the molasses medium. (■, ▲), Biomass (medium without '■' and with '▲' addition of chromic chloride). (●, ×) Ethanol (medium without '●' and with '× ' addition of chromic chloride).

of fermentation in static and semiaerobic conditions was 10 h.

Chromium and protein contents were determined in semiaerobic as well as in static conditions of yeast fermentation biomass. Our aim was to establish the conditions in (semiaerobic or static) which the yeast cells were capable to accumulate more chromium ions. The results are shown in table 1.

Table 1 shows that fermentation conditions influenced the chromium uptake into yeast biomass. The number of chromium ions accumulated into yeast biomass was slightly higher in static conditions than in semiaerobic conditions. The uptake of Cr^{3+} ions into yeast cells depended, by all means, on the cultivation conditions (pH, aeration), which were considered to be the most important parameters of metal ionic translocation, cultivation medium and cultivation time (Hegoczki *et al* 1996).

In general, in an acid pH medium metals exist as free ionic cations, but in an alkaline pH medium the ionic cations precipitate as insoluble hydroxides or oxides. Most heavy metal hydroxides are insoluble. pH-medium at which precipitation occurs varies with different metals and with various oxidation states of the same element. Some metals, for example copper, have more than one valency state and the oxidized state is favoured by high pH. The hydroxides of these oxidized states are less soluble than those of reduced states and precipitate at low pH values. This low pH value generally increases the availability of metal ions, whereas a high pH value decreases this availability (Gadd and Griffiths 1978). The complex media used in this study contain substances making the chromium ion biologically unavailable at pH 6·0. Further more, in an aqueous solution at pH values higher than 6·0, chromium (III) becomes the subject of the olation process which occurs at neutral or basic pH, $Cr–H_2O$ bonds are modified and $Cr–OH^-$ bonds are created, leading to the formation of giant macromolecules (polymerization of hydrated Cr) that precipitate and are thus biologically inert (Ducros 1992). To avoid the problem of chromium (III) olation in complex media the pH 4·5 was selected for further investigation.

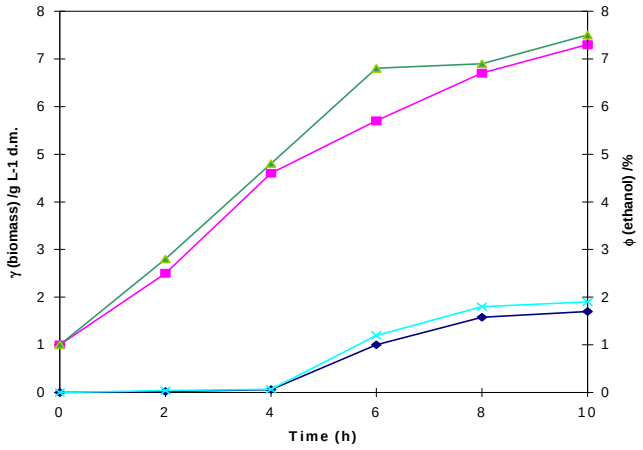

Figure 2. Dynamics of biomass and ethanol production in semiaerobic conditions with 200 mg/l of $CrCl_3$ and without addition of $CrCl_3$ into the molasses medium. (■, ▲), Biomass (medium without '■' and with '▲' addition of chromic chloride). (●, ×) Ethanol (medium without '●' and with '× ' addition of chromic chloride).

Recent studies have shown that chromium biosorption on the cell surface points to a correlation between the pH medium and the chromium accumulation on the cell surface (Batic and Raspor 1998). The metal ions uptake is essentially a biphasic process consisting of a metabolism-independent and metabolism-dependent step. The initial biosorption step for metal ions is rapid, typically only a 5 min in duration, and it is independent of the temperature (White and Gadd 1986; Brady *et al* 1994). The initial binding step is thought to involve the microbial cell surface, and the second, slower step, can be resolved from a metal and enzymatic dependency (e.g. cell wall components-polysaccharides).

The determination of chromium and protein content of yeast cells is important for the fact that GTF in yeast cells has chromium ion binding on a protein molecule.

The yeast biomass was extracted with 0·1 mol/l NH_4OH assuming that the extracted samples had GTF (Mirsky 1993). A few extraction techniques of yeast biomass were developed: extraction with 1 : 1 butanol-water mixture; 0·1 mol/l NH_3 containing 14% *n*-butanol and 0·1 mol/l

Table 1. Chromium and protein content in the yeast biomass.

	Semiaerobic conditions		Static conditions	
	Control sample	Sample with chromium	Control sample	Sample with chromium
w (dry matter)/(%)	18·62	22·56	22·18	23·50
w (proteins)/(%)	60·25	60·71	56·09	59·15
Chromium ($\mu g/g_{d.m.}$ yeast)	2·35	30·93	3·75	43·22

NH_4OH (Anderson *et al* 1978; Mirsky and Weiss 1980; Davies *et al* 1985). In this study the extraction with 0·1 mol/l NH_4OH was selected because in this process we obtained the highest content of chromium and protein. Chromium and protein contents from yeast biomass, which were grown in semiaerobic and static conditions, are shown in table 2.

According to the results in table 2 it is clear that by this method of extraction, most of the chromium material is extractible. More chromium was extracted from a sample of yeast biomass which was grown in semiaerobic conditions, than from yeast biomass which was grown in static conditions.

Anderson *et al* (1978) investigated the optimal conditions of synthesis of a biologically active form of chromium from yeast and the extraction methods for this active product. They extracted yeast cells *S. cerevisiae* with NH_4OH and with ethanol. They investigated the chromium content in these extracts and found that the chromium content was higher in the ammonia extract. Yeast biomass extracts were then passed through a gel-filtration column capable of retaining materials of molecular weight 50000 or less and eluated with H_2O. Collected fractions were measured for optical absorbance at 260 nm (figures 3 and 4)

The measurements were performed at 260 nm because it was observed in a recent study (Beran *et al* 1995) that a maximum appeared at 260 nm in the UV spectra of many components in the yeast extract. This maximum, which is characteristic of heterocyclin nitrogen in an aromatic ring (e.g. in a pyridine ring) is present in fractions with high chromium content. After elution, those fractions (with high chromium content) showed a maximum peak at 260 nm in the UV spectra. Undoubtedly, the presence of nicotinic acid in the complex was responsible for the 260 nm peak. The pattern of biological activity of yeast eluate was very similar to the one with the synthetic material (Toepfer *et al* 1977). Beran *et al* (1995) found that the UV absorption spectra of these materials, concentrated from yeast or a

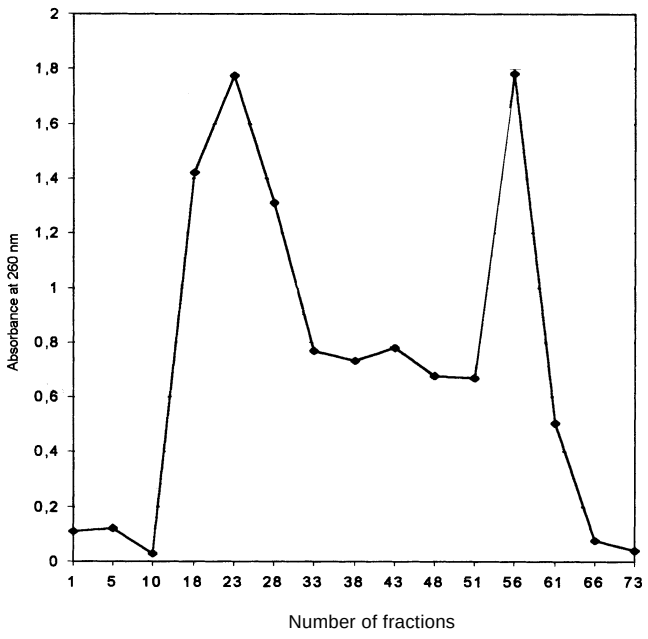

Figure 3. Results of elution of extract from yeast biomass growth in static conditions through Sephadex G-75 column.

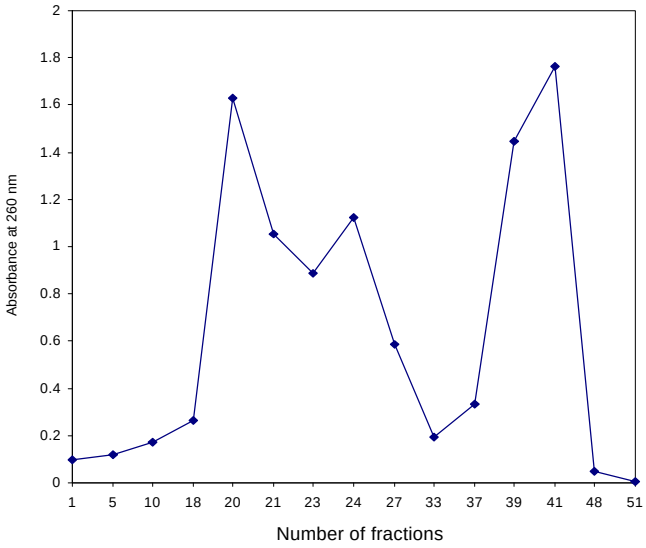

Figure 4. Results of elution of extract from yeast biomass growth in semiaerobic conditions through Sephadex G-75 column.

Table 2. Chromium and protein content of yeast biomass extracts.

	Semiaerobic conditions		Static conditions	
	Control sample	Sample with chromium	Control sample	Sample with chromium
w (dry matter)/(%)	0·30	0·4	0·53	0·65
w (proteins)/(%)	7·46	11·2	8·64	8·83
Chromium *g* (extract)/µg/ml	0·04	7·3	0·16	4·52

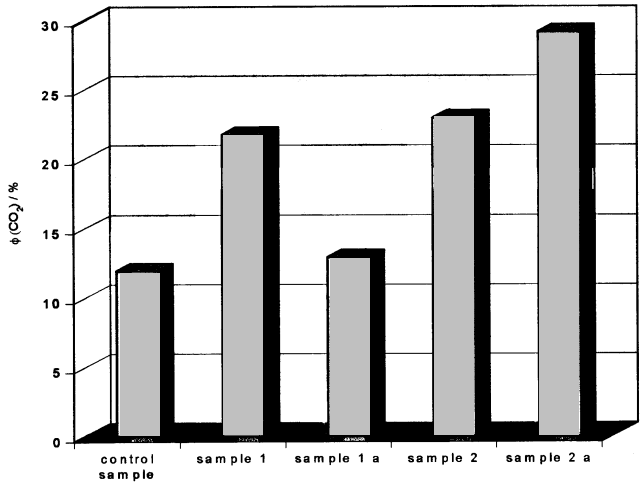

Figure 5. Stimulation of CO_2 production by addition of GTF into the synthetic medium from samples 1, 1a, 2 and 2a. Samples 1 and 1a: GTF in fraction No. 18–51 (sample 1) and No. 52–61 (sample 1a) from yeast biomass extract. Cells growth in static conditions. Samples 2 and 2a: GTF in fraction No. 19–22 (sample 2) and No. 23–27 (sample 2a) from yeast biomass extract. Cells growth in semiaerobic conditions.

synthesized complex Cr^{3+} with *b*-NADP, were almost identical with a maximum at 262 nm. The evidence of an active GTF component in fractions with UV maximum was estimated by CO_2 production with yeast *S. cerevisiae* in static conditions (figure 5) as well as with glucose utilization. Purified GTF which was added in synthetic medium enhanced CO_2 production. For example the amount of CO_2 production was 28% in sample 2a, while in the control sample it was only 12% (figure 5). Thus the simple measurement of CO_2 production can be used as a measure of the GTF activity in the fractions with UV maximum.

On the other hand, these fractions added into the medium enhanced the glucose consumption as well.

As expected, the enhancement of CO_2 production depended on the amount of GTF added to the reaction mixture. The first indication that a high GTF was involved indeed in sugar transport can be seen in figure 5, which shows a direct relation between the enhanced CO_2 production and the added GTF in synthetic medium.

The results clearly indicated that GTF enhanced CO_2 production. The material used in the work reported in this paper as GTF had the biological activity expected of GTF in yeast but nobody has isolated pure GTF yet.

The yeast metabolism can be governed in the desired way. Yeast biomass is a good and inexpensive biomaterial for production of desired preparations with microelements (Mirsky 1993). These results have been supported by several authors (Kompulainen *et al* 1978; Mirsky 1993).

4. Conclusion

On the basis of the experimental results we can conclude that the extracted yeast biomass, enriched with chromium ions has, in the water phase, chromium binding on protein molecule.

The yeasts biomass extracts were passed thuough Sephadex G-75 column and GTF was isolated and purified. The fractions with the maximum absorbance (which is characteristic of pyridine ring) with binding chromium were chosen to prove the active component of GTF by measuring CO_2 production and the kinetics of glucose consumption.

The evidence of the existence of the GTF active component in yeast fractions was the enhancement of CO_2 production and the enhancement of glucose utilization during the fermentation process in a synthetic medium with yeast *S. cerevisiae.*

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