Genetically modified yeasts as a new oral drug delivery system: from in vitro to in vivo validation of the concept

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New strategies to prevent or treat diseases have been focusing on innovative approaches, such as the oral administration of living recombinant microorganisms delivering active compounds directly into the human digestive tract. The feasibility of the approach was evaluated using recombinant *Saccharomyces cerevisiae* expressing a model P450. The survival rate of the yeasts and their ability to convert *trans*-cinnamic acid into *p*-coumaric acid were evaluated in artificial digestive systems simulating human gastrointestinal conditions and in the rat. Both in vitro and in vivo, engineered yeasts showed a high survival rate in the stomach and small intestine and were able to carry out the bioconversion reaction throughout the digestive tract. This study reveals the baker's yeast *Saccharomyces cerevisiae* as a promising host for Biodrug development.

Keywords Biodrug; recombinant *Saccharomyces cerevisiae*; artificial digestive systems; rat; gastrointestinal sac technique; survival rate; bioconversion activity

1. Genetically modified microorganisms as new drug delivery vectors to the human gut

1.1 The Biodrug concept

The development of recombinant DNA technology has allowed the emergence of novel applications such as drug production directly in the human digestive tract [1,2]. This new kind of vector offers several advantages over standard techniques [1]. First, the microorganisms, by protecting the active compounds, can allow the administration of drugs, known to be sensitive to digestive conditions when given in classical pharmaceutical formulations. Second, the regulation of gene expression (e.g. using an inducible promoter) makes it possible to target specific sites throughout the digestive tract (i.e. the absorption or reaction site of the drug) and to control drug release. As a consequence, similar therapeutic effects can potentially be obtained at lower doses [3,4] and the degradation of the active compound by acid or proteases should be avoided upstream from its absorption or reaction site.

Genetically modified microorganisms can either perform a reaction of bioconversion or produce compounds of interest directly in the digestive environment. Bioconversion permits the production of active entities or the removal of undesirable compounds. The active compounds can be secreted in the digestive medium [4], be bound to the cells [5] or accumulate inside the cells and be released in the digestive tract by cell lysis [6].

1.2 Potential applications in human health

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The Biodrug concept involves the use of **orally administered recombinant microorganisms as a new drug delivery route to prevent or treat diseases**. The main potential medical applications in term of bioconversion or production of active compounds are cited below.

1.2.1 Bioconversion

Two main types of medical applications have been considered.

First, genetically modified microorganisms have potential as a biodetoxication system in the gut [1]. The aim is to increase the body's protection against environmental xenobiotics, mainly those present in food (such as pesticides or chemical additives) by ingesting microorganisms expressing phase I (cytochrome P450) or phase II (e.g. glutathione S-transferase -GST-) xenobiotic metabolizing enzymes. Therefore, recombinant microorganisms could be used to prevent multifactorial diseases that have been associated with anomalies in human detoxication processes. For instance, a deficiency in GST-M1 has been correlated with an increased susceptibility to different cancers [7].

Second, recombinant microorganisms could correct errors of metabolism resulting either from gastric or intestinal enzyme deficiencies (e.g. lipase) [8] or organ failure (e.g. by removing urea in case of kidney failure) [9]. The last one could constitute an alternative to current therapy such as renal dialysis, which is time consuming and uncomfortable for the patient.

1.2.2 Production of active compounds

The first (and main) medical application derived from the Biodrug concept is the development of oral vaccines [10]. In that case, modified microorganisms will locally deliver antigens to the digestive mucosa in order to stimulate an immune response and ensure a protection against bacterial [11], viral [12] or parasitic [13] diseases, or being used in the management of food allergy [14]. For several immunological and practical reasons, these new vaccines represent a promising alternative to the traditional injectable ones [15]. In particular, oral immunization is the most efficient way to induce a protective local immune response at the site of pathogen contact. Recently, clinical trials have shown the vaccinal efficiency of different recombinant strains of attenuated *Salmonella typhi* in humans [16].

Other medical applications involve the direct production, in the digestive medium, of various biological mediators, such as cytokines. In particular, new approaches to treating inflammatory bowel diseases (IBD) such as Crohn's disease, celiac disease or ulcerative colitis have been considered [17]. Anti-inflammatory and immunosuppressive therapies are commonly used for the treatment of IBD. However, patients are often subject to unpleasant side effects owing to the high level of the drug in the body (systemic administration) and some of them remain refractory to such treatments. Steidler et al. [4] have investigated the potential of alternative therapeutics and shown the interest of delivering interleukin-10 (IL-10), a strong anti-inflammatory cytokine, in a localised manner by the action of recombinant *Lactococcus lactis* (*L. lactis*). A recent phase I clinical trial has reported that the treatment of Crohn's disease patients with *L. lactis* secreting human IL-10 was safe and allowed a decrease in disease activity [18].

1.3 Bacteria or yeasts

Recombinant bacteria, particularly lactic acid bacteria, have been mostly suggested as potential hosts for this new kind of drug delivery system [19]. However, yeasts can be advantageous over bacteria, especially when a eukaryotic environment is required for the functional expression of human genes. Moreover, the absence of bacterial sequences liable to promote gene transfer to host bacteria can be ensured using the efficient site-targeted homologous recombination machinery of yeasts for introduction of the heterologous gene into the yeast genome. Lastly, yeasts are not sensitive to antibacterial agents, allowing the simultaneous administration of the recombinant microorganisms and antibiotics.

In this study, the common baker's yeast ***Saccharomyces cerevisiae*** (*S. cerevisiae*) was chosen owing to its "generally recognized as safe" (GRAS) status and its easy culture and genetic engineering. *Saccharomyces* spp. have already been used in humans, mainly in the treatment of intestinal functional disorders such as colitis or antibiotic-associated diarrhea [20,21].

2. Evaluation of the feasibility of the Biodrug concept using *S. cerevisiae* as host

2.1 The approach

In the first stages of the Biodrug development, one of the challenges is to evaluate the **viability and heterologous activity** of recombinant microorganisms in the digestive environment. These experiments were conducted with a recombinant model yeast strain using complementary approaches: (i) **in vitro**, in artificial digestive systems simulating human gastrointestinal conditions and (ii) **in vivo**, in the rat.

2.1.1 The recombinant model yeast

The scientific feasibility of the Biodrug concept was evaluated using a *S. cerevisiae* strain expressing a model P450. The cytochromes P450 are membranous enzymes that play a major role in the human detoxication system by metabolizing xenobiotics such as drugs, alcohols, procarcinogens, dyes and pesticides [22]. In the present work, the plant P450 73A1 was chosen as a model for a reaction catalyzed by a P450, owing to the nontoxicity and easy quantification of both its substrate and product. The recombinant strain - named WRP45073A1 - (kindly provided by Dr. Denis Pompon, CNRS, Gif-sur-Yvette, France) expresses the P450 73A1 when grown in the presence of galactose [23]. It catalyses the 4-hydroxylation of *trans*-cinnamic acid (CIN) into *p*-coumaric acid (COU) thanks to the cinnamate 4-hydroxylase (CA4H) activity of the model P450. The heterologous protein was synthesized (induction of CYP73A1 by galactose during the last hours of yeast culture) before in vitro or in vivo experiments.

2.1.2 The in vitro gastrointestinal tract models TIM

The **TIM** (for TNO gastro-Intestinal tract Model) **systems** are the in vitro models that, at present time, allow the closest simulation of in vivo dynamic physiological processes occurring within the lumen of the stomach and intestine of human [24,25]. There are composed of the TIM1 (Fig. 1) which reproduces the stomach and the three parts of the small intestine (the duodenum, jejunum and ileum) and the TIM2 (Fig. 2) which simulates the conditions found in the large intestine.

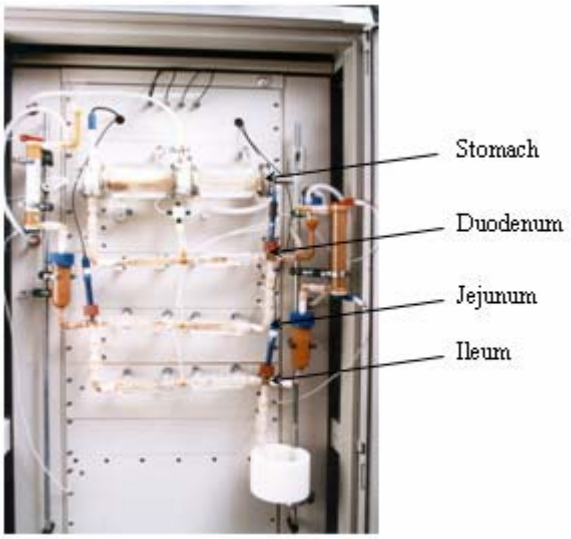

Fig. 1 Gastric and small intestinal system (TIM1).

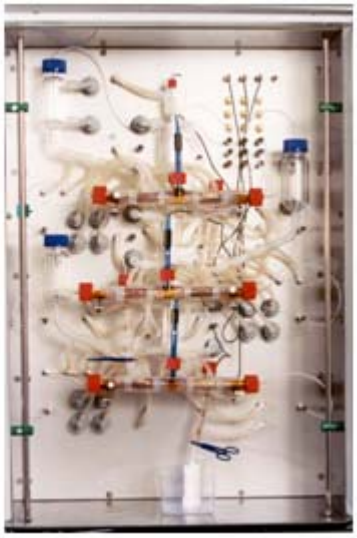

Fig. 2 Large intestinal system (TIM2).

These dynamic, computer-controlled systems have been designed to accept parameters and data from in vivo studies on human volunteers. The main parameters of digestion, such as pH, body temperature, peristaltic mixing and transport, gastric, biliary and pancreatic secretions, passive absorption of small molecules (e.g. nutrients, drugs) and water, and microflora activity are reproduced as accurately as possible (Table 1).

Table 1 Main digestive parameters reproduced in the TIM systems and their simulation.

pH	The pH is computer-monitored and continuously controlled in each digestive compartment. The fall of gastric pH is reproduced by adding hydrochloric acid. The pH is kept to 6.5, 6.8 and 7.2 in the duodenum, jejunum and ileum, respectively, by secreting sodium bicarbonate and to 5.8 in the colon, by secreting sodium hydroxide.
Temperature	The compartments are surrounded by water at 37°C.
Peristaltic mixing	Peristaltic mixing is mimicked by alternate compression and relaxation of the flexible walls containing the chyme, following changes in the water pressure.
Dynamic of chyme transit	A mathematical model using power exponential equations [26] is used to reproduce gastric and ileal deliveries. Chyme transit is regulated by opening or closing the peristaltic valves that connect the compartments.
Volume	The volume in each compartment is monitored with a pressure sensor connected to the computer.
Digestive secretions	Simulated gastric, biliary and pancreatic secretions are introduced into the corresponding compartments by computer-controlled pumps.
Absorption of small molecules and water	Semi-permeable membrane units are connected to jejunal, ileal and colon compartments to passively remove the products of digestion and fermentation, as well as water.

Microflora activity	The colon is inoculated with a human faecal flora and maintained under strictly anaerobic conditions.

Compared with in vivo experiments, these in vitro systems offer accuracy, reproducibility, easy manipulation and the possibility of collecting samples at any level of the digestive tract and at any time during digestion, with no ethical constraint. They have been validated by microbial, nutritional and pharmaceutical studies. For instance, validation experiments demonstrate the predictive value of the TIM systems with regards to the survival rate of probiotic bacteria [27,28], the microflora activity [29], the digestibility of nutrients [30] and the availability for absorption of minerals [31], vitamins [32], food mutagens [33] and drugs such as paracetamol [28,34].

In the present study, the TIM systems were programmed to reproduce gastrointestinal conditions of the human adult after the intake of a liquid meal, according to in vivo data. At the beginning of the experiment, 10^{10} WRP45073A1 cells and 200 µmol of CIN (suspended in yeast culture medium) were simultaneously introduced either in the stomach of the TIM1 or into the TIM2. Digestive samples were regularly collected throughout the artificial digestive tract to evaluate the survival rate of the model yeasts and quantify their CA4H activity.

2.1.3 The in vivo approach in the rat

Experiments were also conducted using **complementary in vivo approaches**: in the living rat, in the different digestive compartments of the sacrificed rat and in ex vivo gastrointestinal sacs.

2.1.3.1 Living rat experiments

The bioconversion activity of yeasts was first followed in the living animal.

Garrait et al. [35] have recently shown that orally administered CIN and COU rapidly travel from the gastrointestinal tract of rats to the bloodstream and are then partially eliminated by the kidney and recovered in the urine. Therefore, after gavage with CIN and the recombinant model yeasts, the CA4H activity inside the digestive tract should be revealed by the apparition of COU in the urine.

Practically, after a 24-h fasting period, rats received a single oral dose of 10^9 WRP45073A1 cells with various amounts of CIN (7, 70 or 700 µmol), suspended in a citrate buffer. The upper extreme of this dose range was the maximum dose tolerated without apparent discomfort to the animals [36]. The yeast heterologous activity in living rats was followed by regularly collecting urine samples before and after gavage of the animals.

Since the search for the appearance of COU in living rats is limited by large losses between the in situ production and excretion of COU in the urine (absorption, body distribution, metabolism and renal elimination), two other in vivo techniques were devised to follow the yeast CA4H activity in the rat digestive tract.

2.1.3.2 Sacrificed rat experiments

This approach offers the advantage to allow for the direct detection of COU potentially produced in the different rat digestive compartments.

Animals were fasted for 24 h and received, by gavage, a single dose of 10^9 WRP45073A1 cells supplemented with 70 µmol of CIN. Thereafter, the rats were regularly sacrificed to collect the different parts of the digestive tract (stomach, duodenum, jejunum, ileum, cecum and colon) and the plasma. The yeast survival rate was evaluated by microbial counting in the mucosal fluid of each organ and in the faeces of rats. The bioconversion activity was followed by measuring COU in the various digestive contents and in plasma samples.

2.1.3.3 Ex vivo experiments

This technique is known to be a useful screening tool for studying the digestive absorption and metabolic behaviour of substances introduced in the various parts of the gastrointestinal tract [37].

After a 24-h fasting period, animals were anesthetized and different parts of the digestive tract (stomach, duodenum, mid-jejunum, ileum, cecum and colon) were quickly removed as described elsewhere [35]. The mucosal and serosal sides of each organ were washed and dried. One end was ligated before introducing the recombinant yeasts (10^9 cells) and 7 µmol of CIN on the mucosal side through the other end. The second end was then ligated and gastrointestinal sacs were immediately transferred to a tissue chamber (Fig. 3) containing warmed (37°C) and oxygenated (95% O_2 / 5% CO_2) Krebs Henseleit modified buffer.

Fig. 3 Tissue chamber contening mid-jejunum preparation.

Samples were regularly collected on the serosal side during 180 min. At the end of the experiment, the gastrointestinal sacs were dried and weighted before collecting the mucosal fluids and the organ walls. The survival rate of recombinant *S. cerevisiae* was evaluated by counting in the mucosal fluids. The yeast activity was determined by measuring the amounts of CIN and COU in the mucosal and serosal fluids and in the organ walls.

2.2 Yeast viability in the digestive environment

Yeast viability was evaluated by microbial counting on a solid selective medium.

2.2.1 Yeast survival rate in vitro

At the end of digestion in the TIM1, yeast survival rate was evaluated by comparing the total ingested yeasts with the living yeasts recovered in both the ileal effluents and the residual digestive content. At 240 min, 79.5 ± 12.1% (n=3) of the ingested WRP45073A1 were recovered alive in the ileal effluents (Fig. 4), leading to a 95.6 ± 10.1% (n=3) survival rate when the yeasts remaining in the residual chyme were added [38]. This high survival rate was confirmed (Fig. 4), no significant difference ($p<0.05$) being observed during digestion between the ileal recovery profiles of the recombinant yeasts and that of a non-absorbable marker, blue dextran, added in the artificial stomach at the beginning of digestion, as previously described [24].

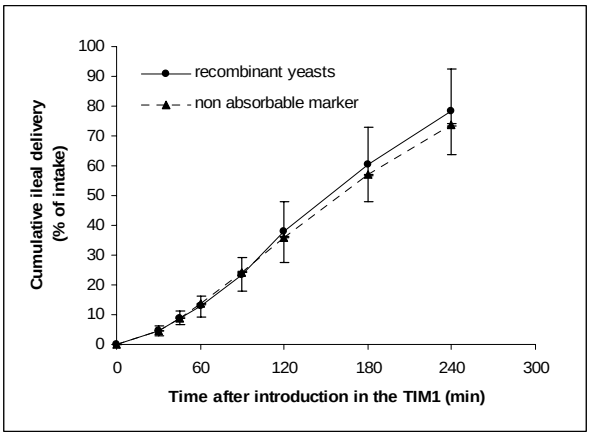

Fig. 4 Yeast survival rate in the gastric and small intestinal system TIM1. The cumulative ileal deliveries of viable yeasts and that of the non-absorbable marker, blue dextran, are represented. Results are expressed as mean percentages ± SD (n=3) of intake.

Yeasts were more sensitive to the large intestinal conditions, only 35.9 ± 2.7% (n=3) of the initial cells remaining alive after 4 h fermentation (Fig. 5). This survival rate decreased to 1.2 ± 0.4% (n=3) after 12 h and no more yeasts could be detected in the artificial colon after 24-h incubation period [38].

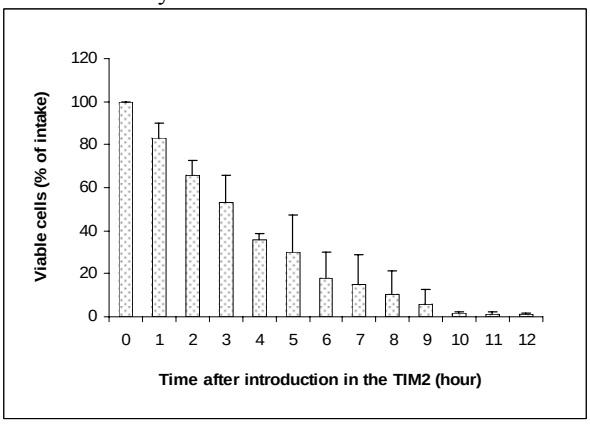

Fig. 5 Yeast survival rate in the large intestinal model TIM2. Results are expressed as mean percentages ± SD (n=3) of viable yeasts relative to the total amount introduced in the large intestinal system.

2.2.2 Yeast survival rate in vivo

In sacrificed rats, no yeast was detected after gavage with physiological water (Fig. 6, control). Thirty minutes after their administration, living WRP45073A1 yeast cells were recovered in all the digestive compartments from stomach to ileum [39], leading to a survival rate of 49.5 ± 12.8% (n=3). Two hours after gavage, the yeasts colonised the large intestine (cecum and colon) and their survival rate decreased to 34.2 ± 8.4% (n=3). The percentages of cells recovered 4 and 8 h after gavage were not significantly different and represented approximately 20% of the total ingested yeasts. At the end of the experiment (24 h), the recombinant cells were only recovered in the feces, accounting for less than 1% of the yeasts initially administered.

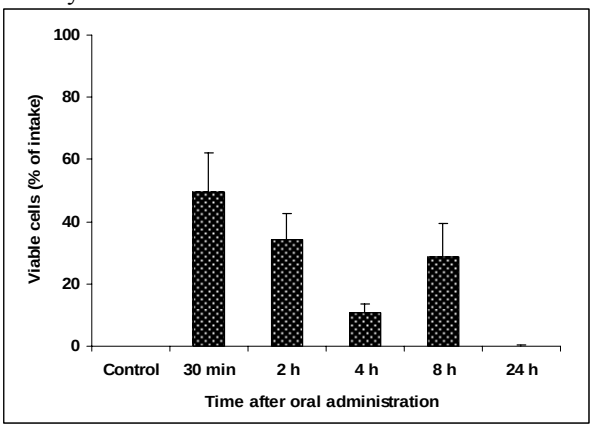

Fig. 6 Yeast survival rate in the rat. Results are expressed as mean percentages ± SEM (n=3) of viable yeasts relative to the total amount administered to rats in the presence of 70 µmol of CIN. Control rats received 0.9% NaCl.

In gastrointestinal sacs, after a 180-min incubation period, about 30% of the initial cells were recovered alive in each organ studied [39]. This survival rate was similar to that observed in sacrificed rats from 2 to 8 h after gavage.

2.2.3 From in vitro to in vivo survival rate

Even if the comparison is hampered by differences in yeast intake and by the fact that the TIM1 and TIM2 are not directly connected, **the survival rates found in vitro in the artificial digestive systems and in vivo in the rat are consistent**. In both cases, WRP45073A1 yeast cells showed a high survival rate in the upper part of the digestive tract (about 100% in vitro vs. 50% in vivo). This result indicates the high resistance of recombinant yeasts to gastric (pepsin and lipase) and small intestinal (bile salts and pancreatic juice) secretions and low gastric pH. According to our results, Gedek and Hagenhoff [40] have shown the survival of a well-known *S. cerevisiae* strain, *S. boulardii*, when incubated during a few hours in artificial gastric juice.

On the contrary, when yeasts reached the large intestine, their survival rates decreased dramatically both in vitro and in vivo (less than 1% 12 and 24 h after ingestion, in vitro and in vivo, respectively).

Few studies have evaluated the viability of *Saccharomyces* spp. throughout the length of the gastrointestinal tract of rats or humans. A similar distribution to that found with WRP45073A1 in sacrificed rats [39] was previously shown by Albert et al. [41] and Bléhaut et al. [42] after the oral administration of 10^9 *S. boulardii.* In the other available studies, the yeast viability has been mainly evaluated in feces. The survival rates observed for the recombinant model yeasts in the feces of rats or in the TIM2 are consistent with those previously obtained for other *S. cerevisiae* strains in the rat [42,43] and in healthy humans [42,44,45].

Several biochemical processes can explain the high mortality of yeasts in the large intestine. Some studies [46,47] have established the prevalence of polysaccharides (glucans and mannans) among the components of the cell wall of yeasts. These polysaccharides can be hydrolyzed by degrading enzymes present in the large intestine. For instance, Salyers et al. [48] have demonstrated the production of β 1–3 glucanases by *Bacteroides*, the most prevalent genus of intestinal bacteria in humans. Moreover, a "barrier" role of the endogenous colonic microflora towards orally administered *S. boulardi* has been demonstrated by Ducluzeau et al. [49] in gnotoxenic mice. This effect induces an extensive elimination of the yeasts from the digestive tract.

Until now, the feasibility of the Biodrug concept had been mainly evaluated with bacteria, especially lactic acid bacteria (see for 1.2). The survival rate of bacteria belonging to this group has also been studied in the TIM1 and in the rat. At the end of digestion in the gastric and small intestinal model, Marteau et al. [27] found a bacterial cumulative delivery from the ileum between 0 and 25% (depending on the tested strain). In the present work, under similar experimental conditions, about 75% of ingested WRP45073A1 were recovered alive. In the rat, Drouault et al. [50] found that 70 to 90% of the ingested *L. lactis* died in the duodenum. The high viability of *S. cerevisiae* in the upper digestive tract might favor the choice of yeasts over lactic acid bacteria as hosts for the development of biodrugs, particularly if the viability of the microorganisms is required for their in situ activity.

2.3 Yeast heterologous activity in the digestive environment

The CA4H activity of WRP45073A1 yeasts was quantified by following COU production by high performance liquid chromatography.

2.3.1 Yeast CA4H activity in vitro

Control experiments with no recombinant yeast showed that both CIN and COU were stable under digestive conditions in the TIM1, whereas they were quickly metabolized by the microflora implanted in the TIM2 [38]. Nevertheless, in all the experiments, in the presence of control yeasts with no CA4H gene

in their plasmid, no COU was produced, showing the specificity of the enzymatic reaction catalysed by the recombinant model yeasts.

At the end of digestion in the TIM1 (240 min), 41.0 ± 5.8% (n=3) of initial CIN were converted into COU (Fig. 7) [38]. Most of the reaction occured within the first 90 min of the experiment.

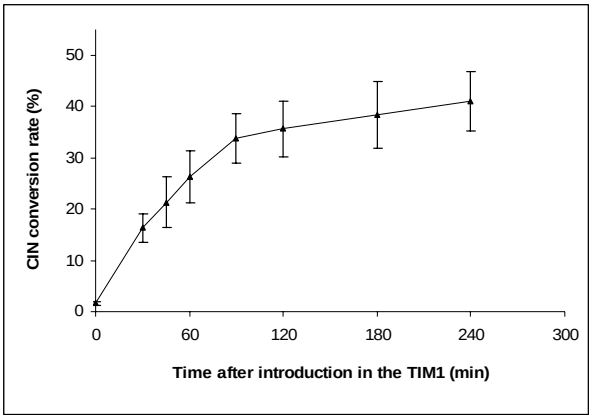

Fig. 7 Yeast CA4H activity in the TIM1. Results are expressed as mean percentages ± SD (n=3) of initial CIN converted into COU in the overall in vitro system.

By means of a computer simulation [38], the CIN conversion rates could be calculated in all the digestive compartments of the in vitro system: 8.9 ± 1.6%, 13.8 ± 3.3%, 11.8 ± 3.4% and 6.5 ± 1.0% (n=3) conversion rates were found in the stomach, duodenum, jejunum and ileum, respectively. These results indicate that the enzymatic reaction occurred throughout the artificial gastrointestinal tract, but mostly in the duodenum and jejunum. This could be explained by the fact that yeasts were no longer stressed by the acid pH of the stomach and could metabolize the CIN that had previously easily entered the cells, owing to the low pH (CIN is essentially in a cationic form which easily diffuses through the yeast membrane [51]). Also, Zarate et al. [52] have demonstrated that bile salts can favor enzymatic reactions. The lower activity in the ileum might result from a decrease in the availability of CIN owing to its previous conversion into COU in the upper digestive compartments. The computer simulation that was developed here should prove useful in future stages of the development of biodrugs, especially if a specific level of the digestive tract has to be targeted for drug action.

Further calculations were performed to quantify the specific enzymatic activity of the WRP45073A1 yeasts in the TIM1. Yeast specific activity (from 0.05 ± 0.04x10^{-10} µmol/cell/min to 3.36 ± 0.86x10^{-10} µmol/cell/min, depending on the digestive compartment and the sampling time [38]) was close to that previously observed in classical batch cultures [23]. This is particularly remarkable as the expression strategy of the model gene had not been yet adapted to the particular constraints of the digestive environment and promising for a future use of recombinant *S. cerevisiae* as vector of active compounds in the upper digestive tract.

The CA4H activity of WRP45073A1 yeasts was also observed in the TIM2 but it was impossible to quantify it. In fact, very small quantities of COU (about 1 µmol) were detected in the colon medium until the complete disappearance of CIN, i.e. after 4-h fermentation [38]. This COU resulted from a balance between that produced from the 4-hydroxylation of CIN by the recombinant yeasts and that degraded by the microflora implanted in the large intestinal model. At the sight of these results, we can speculate that the WRP45073A1 yeasts were metabolically active in colonic conditions during at least 4 h.

2.3.2 Yeast CA4H activity in vivo

Control experiments with the yeasts devoid of the CA4H gene showed the specificity of the enzyme reaction in vivo.

In living rats, COU was detected in the urine only when WRP45073A1 cells and 70 µmol of CIN were administered to the animals. In these conditions, the amounts of COU produced reached 4.5 nmol in 0-2 h and 1.2 nmol in 2-4 h urine samples (Fig. 8), which represent in total 0.007% of the ingested

CIN. When the lowest CIN dose was ingested (7 µmol), the CA4H activity could not be clearly demonstrated because the quantities of COU in urines were too low to be detected [39]. On the opposite, no activity was found with the highest dose (700 µmol), due to an extensive yeast death in the rat digestive tract resulting from the antifungal activity of CIN [53]. Consequently, subsequent experiments in sacrificed rats were performed with 70 µmol of CIN.

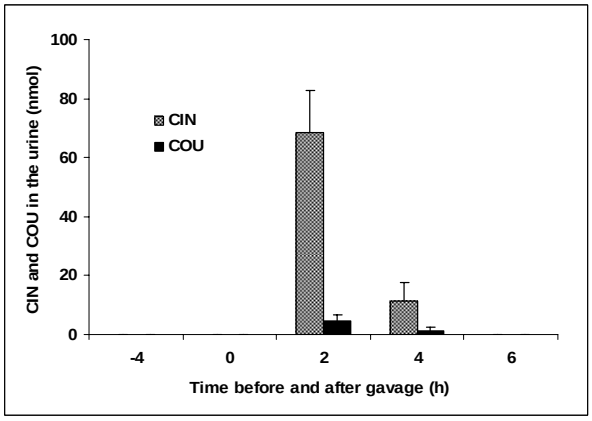

Fig. 8 Yeast CA4H activity in living rats. The values represent the mean amounts ± SEM (n=5) of CIN and COU recovered in urine samples following the ingestion of yeasts and 70 µmol of CIN.

In sacrificed rats, when WRP45073A1 cells and CIN (70 µmol) were orally administered, a CA4H activity was detected, but only in the upper digestive tract. Indeed, small amounts of COU (from 0.3 to 209 nmol) were found in the stomach, duodenum and plasma samples at 5, 10 and 30 min, and in the proximal and median jejunum samples, but only after 5 min [39]. No COU was recovered in the distal jejunum, ileum, cecum or colon of the rat and, whatever the samples, 2, 4, 8 and 24 h after gavage. The bioconversion reaction occured very fast, more than 90% of the total COU being produced within the first 5 min of the experiment. At the end of the assay, 0.83% of the CIN initially administered to rats was converted into COU.

In ex vivo experiments, the CA4H activity was observed in all the digestive organs following the introduction of WRP45073A1 cells and CIN (7 µmol) on the mucosal side of the gastrointestinal sacs. Owing to the absence of systemic circulation (and therefore the limited absorption of CIN), this technique allows the yeast activity to be tested using the lowest nontoxic dose of CIN (7 µmol). The COU produced inside the lumen was absorbed and recovered on the serosal side, where its amount regularly increased with time (Fig. 9). At the end of the experiment, the total amount of COU detected in the three compartments of each organ (serosal and mucosal sides plus organ wall) represented from 2% (duodenum) to 5% (stomach) of the CIN initially administered [39].

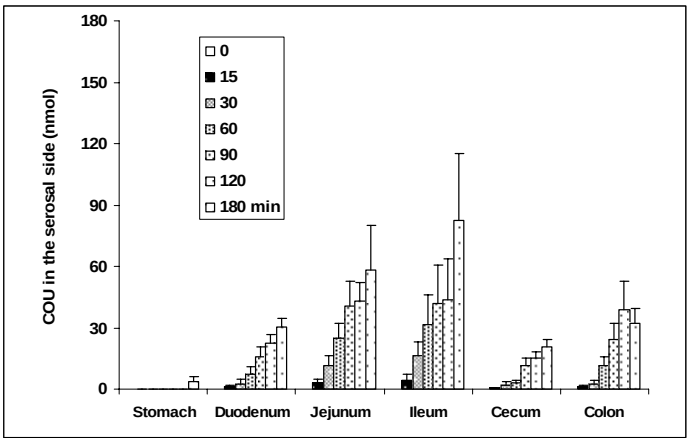

Fig. 9 Yeast CA4H activity in the gastrointestinal sacs. The values represent the mean amounts ± SEM (n=5) of COU recovered on the serosal side of each organ following the introduction of yeasts and 7 µmol of CIN.

2.3.3 From in vitro to in vivo activity

For the first time, **the ability of recombinant yeasts to exert a bioconversion activity in the digestive environment was shown both in vitro, in artificial digestive systems, and in vivo, in the rat**. In the two cases, the reaction occured very fast, COU being detected as soon as 5 min after the introduction of WRP45073A1 cells and CIN in the sacrificed rat and 15 min in the TIM1 and the gastrointestinal sacs. Nevertheless, the in vivo CA4H activity was much lower than that found in vitro : CIN conversion rates of 0.007%, 0.83% and from 2 to 5% were found in the living rat, in the sacrificed animal and in the gastrointestinal sacs, respectively, versus 41% in the TIM1.

This phenomenom is certainly a consequence of the differences existing between the various in vivo and in vitro approaches in term of absorption and metabolism of CIN and COU. The large losses of COU (body distribution, metabolism and renal absorption) in living rats may explain that the amounts of COU recovered inside the digestive tract of sacrificed rats were 100 times higher than those excreted in the urine of the animal. In addition, the CA4H activity observed in ex vivo experiments was some 7 times higher than that found in sacrificed rats. This could be linked to a faster and more extensive absorption of CIN in the sacrificed animal, due to its distribution to the whole body by the systemic circulation. As a consequence, the recombinant yeasts were quickly deprived of their substrate, resulting in a lower CA4H activity. We can suppose that a closely similar effect was observed in the TIM1 in which CIN was exclusively absorbed from the jejunum and ileum via passive diffusion and not via a monocarboxylic acid transporter as it had been previously shown in the rat gastrointestinal sacs [35] or in Caco-2 cell monolayers [54]. In the gastric and small intestinal system TIM1, the yeasts were therefore in close contact with their substrate for a longer time, enhancing their activity.

No clear relation could be established between in vivo and in vitro results with regard to the digestive compartments where the highest yeast activity was found. In vitro, the highest CA4H activity was found in the duodenum and jejunum, whereas in vivo it was in the stomach and jejunum. Moreover, the yeast bioconversion activity could be quantified in ex vivo experiments in the cecum and colon of rats (CIN conversion rate of about 2-3%) but not in the large intestinal model. The reason is that, in the TIM2, the recombinant yeasts were rapidly deprived of CIN due to its metabolization by the colonic microflora whereas most of the rat endogenous flora was removed from the mucosal side of the gastrointestinal sacs by rinsing during their preparation.

The recombinant yeast used to validate the Biodrug concept in term of bioconversion is less well tailored for in vivo experiments than for in vitro ones, due to the extensive and rapid absorption of CIN by the rat gastrointestinal tract. Nevertheless, both in vitro and in vivo results showed the yeast ability to perform CA4H activity throughout the gastrointestinal tract, supporting the possibility of using genetically modified *S. cerevisiae* as a potential host for the development of biodrugs.

3. Conclusion and future developments

Using genetically engineered microorganisms as new delivery vehicules to the gut is an important challenge for the development of innovative drugs. A potential application directly issued from the present work is the development of drug delivery systems based on orally administered yeasts carrying out a bioconversion reaction. In particular, they could perform biodetoxication by metabolizing xenobiotics that are poorly or slowly absorbed by the human digestive tract.

Soon, once a therapeutic target identified, model genes will be replaced by candidate genes. Of course, heterologous gene expression strategies have to be tailored for a safe use in human, the presence of mobilisable vectors, antibiotic selection markers and bacterial sequences liable to promote gene transfer to host microflora being prohibited. In addition, environmental confinement of recombinant cells has to be achieved by introducing a suicide process that triggers the elimination of the yeasts upon leaving the digestive tract. Two types of biological containment systems may be considered : (i) an active system which should provide a control of the dissemination of recombinant microorganisms through the conditional production of a toxic protein [55] and (ii) a passive system that could render the cell growth dependent on the complementation of an auxotrophy or other gene defects [56]. Steidler et al. [57] have

already developed and validated in pigs a passive containment system for the *L. lactis* expressing human IL-10 by deleting the thymidylate synthase gene which is essential for their growth. Oral formulations will have also to be developped [58,59] in order to (i) control the release of yeasts according to their action site in the gastrointestinal tract, (ii) maximize the heterologous activity of yeasts (by addition of the appropriate substrate and/or inductor) and (iii) ensure a stability of both yeasts and pharmaceutical dosage forms before administration to patient.

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