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D-TAGATOSE PRODUCTION FROM D-GALACTOSE BY IMMOBILIZED L-ARABINOSE ISOMERASE FROM THERMOTOGA MARITIMA

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27 maggio 2013

To my angels
Lorenza and
Filippo

Contents

Abstract

D-Tagatose, an isomer of D-galactose, is a rare ketohexose which can potentially be used as a sugar-substitute bulking agent in food and as a reduced energy sweetener. Furthermore, D-tagatose has numerous medical benefits and is being considered as a drug for type II diabetes. The enzymatic isomerization of D-galactose to D-tagatose represents a potential alternative to the recently patented chemical production process of D-tagatose. The enzyme L-arabinose isomerase is able to catalyze this reaction. In particular there is interest in its thermostable forms since the equilibrium is shifted toward D-tagatose at high temperatures (T>60°C). This work was an investigation of the enzyme L-arabinose isomerase (TMAI) from the hyperthermophilic bacterium *Thermotoga maritima*, immobilized on epoxy supports for the bioconversion of D-galactose to D-tagatose. The enzyme, as a recombinant protein fused to a His-tag, was expressed in *E. coli* and produced in 2-liter stirred bioreactors. TMAI extract, a partially purified solution containing TMAI obtained after precipitating heat-denatured proteins following heat treatment (80°C, 20 min) of lysate supernatant, was used for immobilization. A further purification step by IMAC, taking advantage of the His-tag fused to the protein, was used to obtain a purified enzyme to be studied as free enzyme. Epoxy supports (Eu), as such or functionalized with iminodiacetic acid and chelated with copper ion (Eu-Cu) or manganese ion (Mn-Cu) to increase the selectivity of the immobilization process were used for the immobilization starting from the TMAI extract. The best results for the immobilized biocatalysts, characterized in terms of immobilization yield, specific activity and catalytic efficiency determined at 80°C and pH 7.5, were obtained for Cu-Eu supports and were respectively 33.9 mg of TMAI per g ds (dry support), 5.4±1.1 U/g ds and 99±20%. The specific activity of the biocatalyst determined at pH 7.5 decreased 5 times when the temperature was decreased from 80 to 60°C. The immobilized enzyme, incubated at 80 and 90°C in absence of D-galactose for different times, exhibited a higher half-life compared to the free enzyme. However, in the presence of D-galactose, that is under operational conditions, the immobilized enzyme exhibited a lower stability compared to incubation in absence of D-galactose. A screening of three buffer systems each at two levels of pH in activity experiments at 80°C with D-galactose over long incubation times identified Tris-HCl buffer at pH 7.5 as the best system. The effect of different post-immobilization treatments consisting of incubating the immobilized derivatives under conditions that should favour stabilization of the enzyme was studied: combined use of glutaraldehyde and

ethylenediamine, high temperature (50°C), high pH (10) and long incubation time (72 h), use of glutaraldehyde. The treatment that resulted in the best operational stability of the immobilized biocatalyst was the one with glutaraldehyde and ethylenediamine. In particular, this treatment was also able to stabilize the quaternary structure of the enzyme, a result that was not obtained when applying the other treatments. A further treatment with mercaptoethanol on the biocatalysts, stabilized with the best method or non stabilized, further increased the specific activity, most likely because it was able to remove the Cu^{2+} ions from the support, thus avoiding its release in the bioconversion medium and its potential negative effect on the enzyme activity. The results obtained showed that the combined use of different strategies, such as use of enzyme tags, functionalization of epoxy supports, use of a hyperthermophilic enzyme and use of post-immobilization methods for stabilization offers great advantages over other methods in the development of an immobilized-enzyme biocatalyst. Recent studies on D-tagatose bioproduction from D-galactose are focusing on the use of thermophilic enzymes with optimum pH on the acidic side. Another strategy is the use of thermophilic or hyperthermophilic enzymes at temperatures in the range 55-65°C in order to avoid side reactions and deactivation of the enzyme. The use of the methods tested in this work on TMAI coupled with the abovementioned strategies could further improve the operational enzyme stability for D-tagatose isomerisation at high temperatures making this process feasible at the industrial scale.

1. Rare sugars

1.1 Definition of rare sugars

"Rare sugars" are sugars that occur naturally in extremely small amounts, usually because they are the products of intermediary cellular metabolism and are converted within the cell to another form as soon as they are created, and include D/L-allose, D/L-altrose, L-glucose, D/L-psicose, D/L-tagatose, L-fructose, and D-sorbose. According to the International Society of Rare Sugar (ISRS) (Granström *et al.*, 2004) established in Japan, rare sugars are monosaccharides and their derivatives that are rare in nature and oligo- and polysaccharides containing rare sugars. Thus, this definition includes also the polyols, also known as sugar alcohols. As shown in Fig. 1, among six carbon sugars consisting of 34 hexoses (8 ketohexoses, 16 aldohexoses, and 10 hexitols), the only carbohydrate monomers that occur in large amounts in nature are D-glucose, D-galactose, D-mannose and D-fructose while among five carbon sugars consisting of 16 pentoses (8 aldopentoses, 4 ketopentoses, 4 pentitols), D-xylose, L-arabinose, and D-ribose are the only monosaccharides abundant in nature.
Figure 2 and Figure 3 report the structure of the D form of ketoses and aldoses, respectively, containing 4, 5 and 6 carbon atoms.

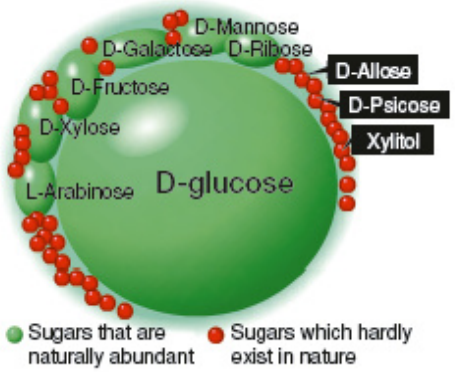

Figure 1: Distribution of the amount of monosaccharides and their derivatives on earth. Sugars shown in green exist in large quantities while rare sugars are represented by small red circles (http://rare-sugar.com/en/research3e.html).

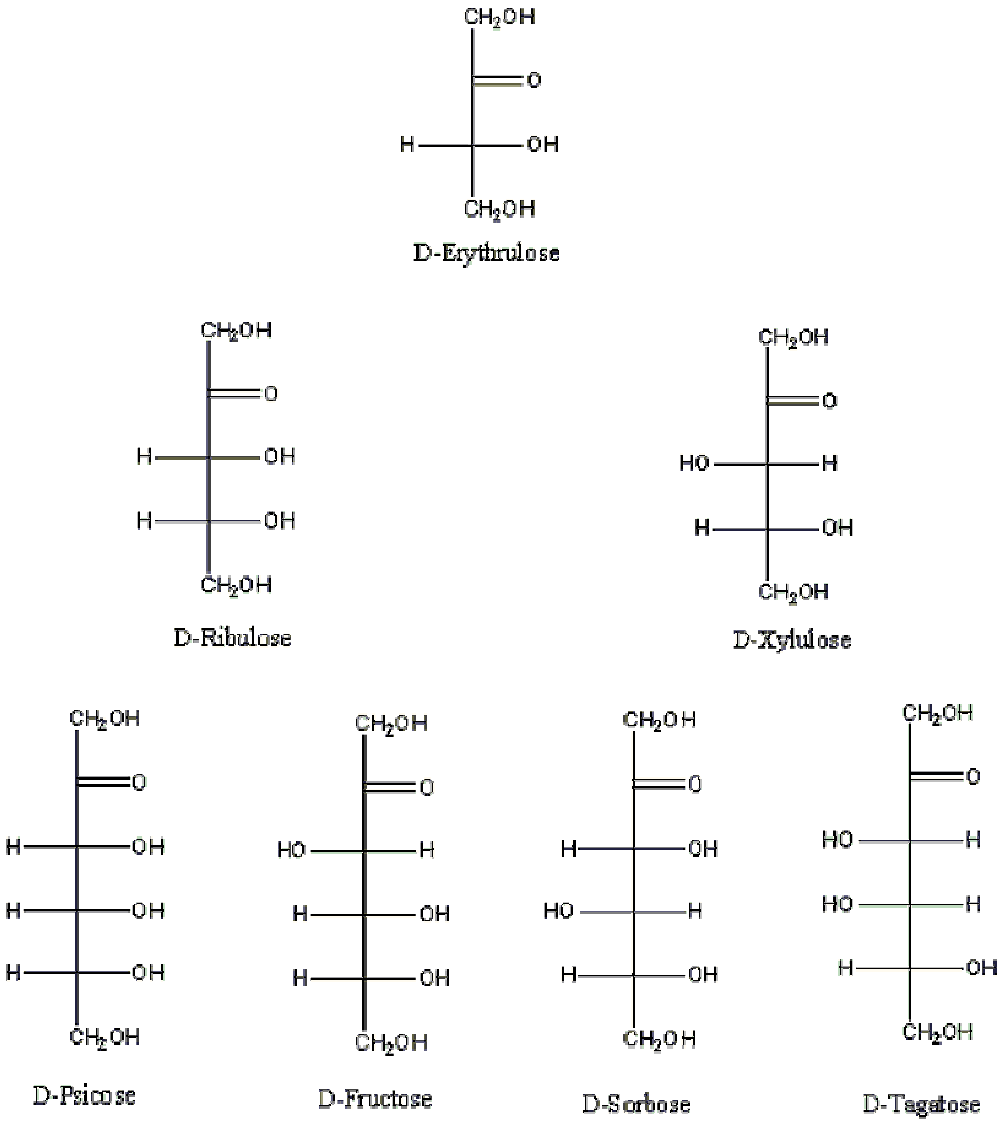

Figure 2. D form of 4-carbon, 5-carbon and 6-carbon ketoses.

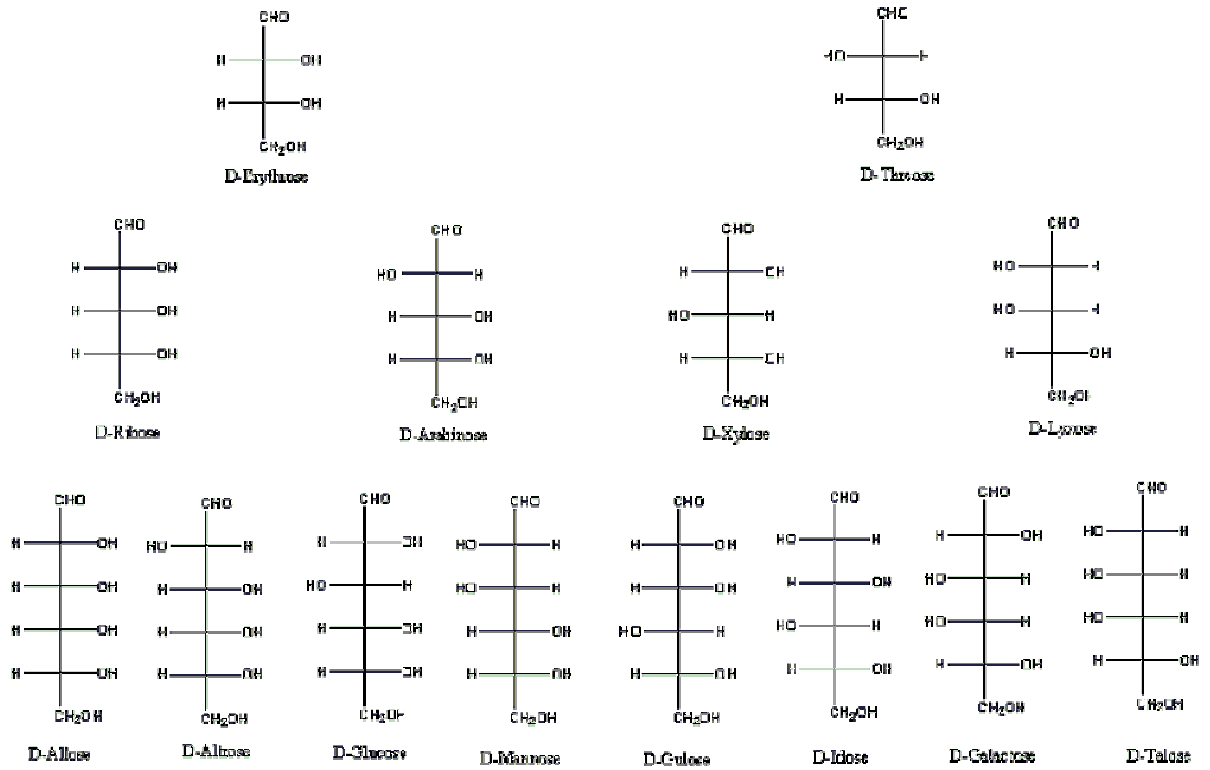

Figure 3. D form of 4-carbon, 5-carbon and 6-carbon aldoses.

1.2 Applications of rare sugars in the food industry

Because until now rare sugars have only been accessible in very small amounts in nature, their properties have not been evaluated. Until recently, research both on methods for the production of substantial quantities of rare monosaccharides and studies of their potential use have been neglected. Expected application fields and products for rare sugars are:

(1) Foods and beverages, sweeteners, seasonings;
(2) Foods for specified health uses, healthy foods and beverages;
(3) Agricultural chemicals that induce resistance against diseases, control of plant growth;
(4) Pharmaceuticals, organ conservation solutions;
(5) Use as high value building block for the preparation of pharmaceutical products.

1.3 Use of sugars and rare sugars as sweeteners

Sucrose and fructose, among the most abundant sugars in nature are primary sugar sweeteners that occur naturally or are added to foods. In addition to their sensory qualities, they add functional properties to foods through their effects on physical (eg, crystallization, viscosity), microbial (e.g., preservation, fermentation), and chemical (e.g., caramelization, antioxidation)

characteristics (Davis, 1995). Sucrose is a disaccharide composed of glucose and fructose that provides 4 kcal/g. The monosaccharide fructose also provides 4 kcal/g. Fructose is a component of sucrose (50% fructose), is present in fruit (also known as fruit sugar or levulose), and is added to foods and beverages as high fructose corn syrup (HFCS; 42% to 55% fructose) or in the crystalline form. Fructose has replaced sucrose in many foods and beverages because of its sweetening power, lower cost, and functional properties that enhance flavor, color, and product stability (Hanover and White, 1993). Fructose also synergizes the sweetness potential of sucrose and certain sweeteners.

Polyols are used as sugar substitutes for their sweetening power. Sugar alcohols have less energy per gram (averaging 2 kcal/g) compared to sucrose for example, because they are not fully absorbed from the gut and thus are less available for energy metabolism. Table 1 provides a list of the approved polyols and novel sugars. In addition to containing less energy than sugars, they have other potential health benefits (i.e., reduced glycemic response, decreased caries risk, prebiotic effects). Polyols can have a chemical structure that is monosaccharide-derived (eg, sorbitol, mannitol, xylitol, erythritol), disaccharide-derived (eg, isomalt, lactitol, maltitol), or polysaccharide-derived mixtures (eg, maltitol syrup, hydrogenated starch hydrolysates [HSH]). Sorbitol is on the GRAS list for use in candies, chewing gum, jams/jellies, baked goods, and frozen confections.

Mannitol is permitted for use on an interim basis pending further study of health effects, including potential laxative effects. This status is provided to food ingredients that have a history of use but whose safety has been brought into question by new information, even if it is not conclusive (FDA, 1996). Mannitol is used as a dusting agent for chewing gum and a bulking agent in powdered foods. Xylitol is approved as a food additive for use in foods for special dietary use. The polyols offering reduced-calorie sweetening are absorbed slowly and incompletely from the intestine by passive diffusion. An excessive load (eg, greater than 50 g/day of sorbitol; greater than 20 g/day of mannitol) may cause diarrhea. However, incomplete absorption causes indirect metabolism via fermentative degradation by intestinal flora.

Glycemic responses are lower for sweeteners that undergo incomplete absorption. The potential for prebiotic effects of sweeteners that undergo incomplete absorption is being explored. These prebiotics are short-chain carbohydrates that are resistant to human digestive enzymes and reach the cecum to exert effects on the colonic bacteria (Van Loo *et al.*, 1999). Because of their chemical structure, polyols could serve as a substrate for these bacteria (Cummings *et al.*, 2001).

Worldwide polyalcohol use is about 1.2 million tons/year. Sorbitol has the greatest market among the polyalcohols (around 1 million tons/year) with the cheapest price competitiveness among the polyalcohols: US $1.2/kg. Other polyalcohols, such as xylitol, erythritol, mannitol, and isomaltol, are consumed at 200,000 tons/year and have a higher price range (US $3–7/ kg) (Kim, 2004).

1.4 Some rare sugars of potential application in the food industry

Low caloric alternatives to the present dominant sweeteners are being sought due to the increasing number of people suffering from diabetes, obesity, cancer and cardiovascular diseases. Some isomers of monosaccharides, for example L-sugar or D-tagatose, have been proposed as alternative carbohydrate sweeteners and bulking agents as sugar substitutes because of the disadvantages of sucrose intake, namely, lipogenic potency, and high insulinogenicity. (Zeng *et al.*, 2005).

1.4.1 Xylitol

Xylitol is one of most significant alternatives to conventional sweeteners and is the first rare sugar with health properties that has global markets. Xylitol is a five-carbon sugar alcohol that can be found in nature in small quantities, for example in fruits and vegetables. Xylitol has almost the same sweetness as sucrose, but with an energy value of only 2.4 kcal/g. Xylitol has a negative heat of solution and good solubility in water, causing a cooling sensation when consumed orally (Granström *et al.*, 2007).

It was approved by the US Food and for use in food products back in the 1960s. It has since experienced remarkable growth following its commercialisation in the early 1970s C. In 2002, its worldwide annual production was estimated to be about 15,000 t.

Xylitol is industrially produced by the chemical reduction of pure D-xylose, obtained from hard-wood hydrolysates, in the presence of a Raney nickel catalyst (Granström *et al.*, 2007).

Xylitol production with yeasts has been studied extensively as an alternative to the chemical reduction process (Granström *et al.*, 2004). In fully aerobic conditions D-xylose is used for cell respiration and biomass growth, but under oxygen limited conditions the yeast cells produce xylitol. The advantage of using whole cells instead of chemical reduction in xylitol production lies in the fact that crude industrial side streams could be used as a raw material. Even though part of the D-xylose goes into cell maintenance metabolism, the xylitol yield is significantly higher in the biotechnological process than in chemical reduction.

Table 1. Polyols and novel sugars approved by FDA: E (energy intake), from ADA (American Dietetic Association) report (2004).

Type	E kcal/g	Regulatory status	Description
Monosaccaride polyols or novel sugars			
Sorbitol	2.6	GRAS-Label must warn about a laxative effect	50-70% as sweet as sucrose; some individuals experience a laxative effect from a load of ≥50g.
Mannitol	1.6	Approved food additive; the label must warn about a laxative effect	50-70% as sweet as sucrose; some individuals experience a laxative effect from a load of ≥20g.
Xylitol	2.4	Approved food additive for use in food for special dietary uses	As sweet as sucrose; new forms have better free-flowing abilities.
Erythritol	0.2	Independent GRAS determination; non questions from FDA	60%-80% as sweet as sucrose; also acts as a flavor enhancer, formulation aid, humectant, stabilized and thickener, sequestrant and texturized.
D-tagatose	1.5	Independent GRAS determination; non questions from FDA	75%-92% as sweet as sucrose; sweetness synergized; functions also as textutixer, stabilizer, humectant, and formulation aid.
Disaccharide polyols or novel sugars			
Isomalt	2	Gras affirmation petition filed	45%-65% as sweet as sucrose; used as a bulking agent.
Lactitol	2	Gras affirmation petition filed	30%-40% as sweet as sucrose; used as a bulking agent.
Maltitol	2.1	Gras affirmation petition filed	90% as sweet as sucrose; used as a bulking agent.
Trehalose	4	Independent GRAS determination; non questions from FDA	45% as sweet as sucrose; functions also as a texturizer, stabilizer, and humectant.
Polysaccharide polyols			
HSH	3	Gras affirmation petition filed	25%-50% as sweet as sucrose (depending of the monosaccharide composition)

Xilitol is mainly used as an additive in commercial products such as chewing gums, mints, sweets, and tooth paste. It has an insulin independent metabolism, and it can prevent dental caries and acute otitis media in children (Granström *et al.*, 2004)

1.4.2 Xylitol in dental caries

Dental caries is a highly prevalent disease in human race. One recommendable preventive measure is not to eat sugars such as sucrose that generate insoluble glucans or acids by the actions of oral bacteria. However, as sucrose is indispensable for our diet as a seasoning and food component, its intake is very difficult to avoid. Xylitol is widely known to be used as an additive for foods and sweets and to produce a marked preventive effect against caries.

A study, published online in the journal BMC Oral Health, evaluated the impact of incorporating xylitol, already linked to fighting the harmful *Mutans streptococci* bacteria, in the traditional confectionery product. Head researcher Kiet Ly of the University of Washington (WA, USA) said that controlled doses of xylitol containing gummy bears could be a more ideal solution than chewing gum for delivering the sugar alcohol to children as xylitol is a cariostatic bulk sweetener (Ly *et al.*, 2008).

1.4.3 D-psicose

D-psicose, a C-3 epimer of D-fructose, is present in small quantities in commercial mixtures of D-glucose and D-fructose, which is obtained from hydrolysis of sucrose or isomerization of D- glucose. Supplement of D-psicose in the diet has been found to suppress hepatic lipogenic enzyme activities in rats compared with supplements of D-fructose or D-glucose and provide zero energy. D-Psicose might be an ideal sucrose substitute for food products due to its sweet taste, easy processing, and functional properties (non caloric and low glycemic response) (Yun *et al.*, 2008). It might be useful in the food industry as non-caloric sweetener for obese people as an aid for weight reduction (Zeng *et al.*, 2005).

The safety of D-psicose, mass production of which has become possible, must be confirmed before it can be used as a food material. When the D-psicose contents of various foods were measured to evaluate the routine D-psicose intake level (Oshima *et al.*, 2006), its contents were particularly high in sweets and seasonings, indicating that we are regularly taking D-psicose from various food materials (Figure 4).

D-Psicose was shown experimentally (in rats and humans) to suppress increases in the blood sugar level. Inhibition of digestive enzyme activities and promotion of glycogen synthesis

have been suggested as mechanisms of this effect (Matsuo *et al.*, 2002). Therefore, D-psicose may be useful as a functional food.

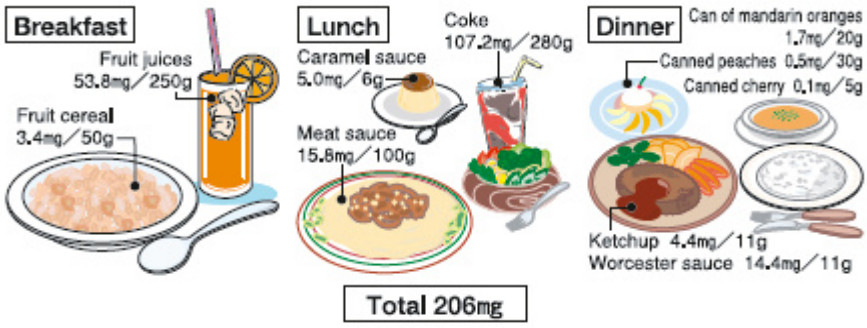

Figure 4. Daily D-psicose intake from various foods (Oshima *et al.*, 2006).

1.4.4 D-psicose in dental caries

Currently scientists are examining the effects of some rare sugars on the proliferation of bacteria responsible for caries, acid production, insoluble glucan formation, etc. by adding them to the culture medium of the bacteria.

Results indicate that some rare sugars are poorly or scarcely metabolized by *S. mutans*, which causes dental caries. In particular D-psicose practically controls the bacterial growth and acid production (anti-cariogenic) also in combination with existing sweeteners (Iida *et al.*, 2010). Table 2 reports comparison of D-psicose and xylitol as sugar substitutes. The authors of this research anticipated the use of D-psicose in the field of drugs, foods and beverages, foods for specified health uses and cosmetics.

Table 2. Comparison of D-psicose and xylitol as sucrose substitutes.

Sugar	Use as a substitute for glucose and fructose	Risk of diarrhea by massive intake	Sweetness	Cost
D-psicose	Wide	Low	Weak	High
xylitol	Narrow	High	Same as sucrose	Low

1.4.5 D-psicose and D-allose as improving of food quality

Surimi, a water-leached and cryo-stabilized ground fish muscle paste, is a raw material for making gelled seafood products, such as kamaboko and imitation shellfish. Surimi proteins denature during freezing and frozen storage, leading to significant deterioration in the quality

of final gelled seafood products. Currently, Prof. Ogawa from Kagawa University (Japan) is investigating whether the rare sugars D-psicose and D-allose prevent surimi proteins from denaturing by freezing and frozen storage. D-psicose and D-allose were found to prevent the solubility of fish muscle proteins from reducing during frozen storage, similarly to D-sorbitol (sugar alcohol), a conventional cryoprotectant. This effect was particularly high with D-psicose. D-psicose was found to improve the elasticity of fish muscle proteins, which is an index of the surimi quality, and to prevent the decline in the gel-forming ability of surimi occurring during its frozen storage.

If the cost of D-psicose is reduced to about 1000 yen/kg, its application to surimi-based products will become possible.

1.4.6 D-psicose and D-allose as healthy foods

The Maillard reaction is well known for the many changes it produces in food. This non-enzymatic browning occurs spontaneously when reducing sugars, such as glucose, are incubated with protein over time. The carbonyl groups of reducing sugars react with protein amino groups and lead to irreversible changes in the conformation and stability of the protein. The resulting reaction products can potentially alter the functional and/or the biological properties of food. Maillard reaction products (MRPs) are formed following heat processing of food and the reaction products have some beneficial effect on food quality. The current trend is to use MRPs as commercial food additives in applications as emulsifiers, antioxidants and antibacterial agents (Puangmanee *et al.*, 2008).

Proteins that are glycated with rare sugars have been found to have high antioxidant activity and also show some improved functional properties compared to native proteins or proteins conjugated with conventional sugars. Glycated whey protein isolate (WPI) was prepared by incubation of WPI with the rare sugars D-allose and D-psicose under controlled conditions (Puangmanee *et al.*, 2008). Its emulsion and foaming properties, and its antioxidant activity were determined and compared to those of the native WPI and of WPI glycated with the alimentary sugars D-glucose and D-fructose. WPI glycated with ketohexose showed a greater ability to form emulsions and a higher foam stability than control WPI or WPI glycated with aldohexoses. Moreover, WPI glycated with the rare sugars D-allose and D-psicose had the highest antioxidant activity even at low concentration. WPI glycated with rare sugars was then applied to ice cream manufacture and the resulting ice cream properties were evaluated and compared with ice cream made from skim milk powder (SMP). The ice cream made with added glycated WPI showed ice cream overrun and hardness that was intermediate between

that of SMP and native WPI ice cream. On the other hand, the modified ice creams containing WPI glycated with the rare sugars D-allose and D-psicose had significantly higher antioxidant activity than the other ice cream samples. Thus, glycation of WPI with D-psicose improved emulsion and foaming properties and, after application in ice cream manufacture maintained high antioxidant activity. Fortification of ice cream with glycated WPI can therefore produce ice cream with excellent antioxidant activity and good ice cream qualities.

Low-calorie and highly antioxidative foods can be prepared by adding the rare sugar D-psicose to bakery products, such as pudding. Pudding prepared by the addition of D- psicose showed high antioxidative activity and excellent food properties, and is promising as a functional dessert for elderly people (Sun *et al.*, 2007).

The addition of D-psicose to butter cookies, as partial replacement of sucrose, had no influence on the cook loss while significantly contributing to a color change of the cookie crust through a non enzymatic browning reaction. Furthermore, D-psicose-containing cookies possessed the highest antioxidant capacity in all tested cookies. It was found that there was a close correlation between the crust color and the antioxidant activity of the cookie. The results suggested that the addition of D-psicose enhanced the browning reaction during cookie processing and, consequently, produced a strong antioxidant activity (Sun *et al.*, 2008).

1.4.7 Erythritol

Erythritol is a polyol that occurs at low levels in many fruits and at higher levels in fermented foods such as soy sauce, cheese, wine and beer. It is used to reduce or replace traditional sweeteners in low-sugar food and beverage applications and the benefits include low-calorie content, low glycemic index and a low laxative effect. In Europe, despite its low energy content, regulations indicate that it cannot be labelled as a low calorie because it is classified as a polyol (a sugar alcohol). Erythritol is a white, crystalline, odorless powder and is approximately 70 percent as sweet as sucrose with a clean, sweet taste similar to sucrose. At 0.2 calories per gram, erythritol has approximately 7 to 13% the calories of other polyols and five percent the calories of sucrose. Erythritol has been allowed for use in the US since 1997 where it can be described as natural and low-calorie. Erythritol and erythritol-containing sweetening systems produced by the company Corn Products Specialty Ingredients (USA) come under the brand *Erysta* and the company said it can be used alone or in combination with other polyols and sweeteners (Hills, 2008).

1.4.8 D-tagatose

D-Tagatose is a ketosehexose monosaccharide sweetener. D-Tagatose occurs naturally in *Sterculia setigera* gum, a gum exudate of the cacao tree (Hirst *et al.*, 1949). D-tagatose was also detected ad a component of an oligosaccharide in lichens of the Rocella species (Lindberg, 1955). In bacterial metabolism of lactose, D-tagatose may be formed from galactose by enzymatic isomerization (catalyzed by L-arabinose isomerase) (Szumilo and Russa, 1982; Ibrahim and Spradlin, 1993). D-tagatose is also found in small quantities in various foods such as sterilized and powdered cow's milk, hot cocoa, and a variety of cheeses, yogurts, and other dairy products (Mendoza *et al.*, 2005; Oh, 2007).

D-Tagatose has unique properties as a functional sweetener (Levin, 2002). It is an isomer of D-galactose (Fig. 2 and Fig. 3). Its properties are reported in Table 3.

Table 3. Physical, chemical and biological properties of D-tagatose as a sweetener (Kim, 2004).

Property	**Value**
Chemical family	Carbohydrate ketose monosaccharide, isomer of D-galactose
Molecular weight	180
Physical form	White crystalline solid
Odor	None
Melting point	143 ^{o}C
Solubility	58% w/w 21^{o}C
Stable pH range	2-7
Relative sweetness	92% of sucrose in 10% (w/w) solution
Sweetness quality	Similar to sucrose with faster onset like fructose
Cooling effect	None
Caloric value	1.5 kcal/g
Maillard reaction and caramelization	Yes
Potential field of application	Low- calorie dietary food, diabetic food, adjuvant
Regulation state	GRAS approved as drug, animal feed, food (reported range: 30 g/d for healthy male adult)

The cyclic form of D-tagatose consists of α-D-tagato-2,6-pyranose (79%), β-D-tagato-2,6-pyranose (14%), α-D-tagato-2,5-furanose (2%), and β-D-tagato-2,6-furanose (5%) (Oh, 2007). No cooling effect is found, which is a unique property of polyalcohol sugar substitute candidates. Since tagatose is a reducing sugar, it is involved in browning reactions during heat treatment (i.e., cooking processes) (Kim, 2004). D-Tagatose is similar to the polyols in having a low caloric value and tooth-friendly property. However, it has no laxative effect unlike polyols (Levin *et al.*, 1995). The sweetness profile of D-tagatose is 92% that of sucrose when compared in 10% solutions, but its sweetness is detected slightly sooner than that of sucrose. D-Tagatose is less hygroscopic than fructose, and it has a lower viscosity [180 cP at 70% (w/w) and 20°C] than sucrose at the same concentration but slightly higher than fructose and sorbitol.

D-Tagatose has numerous health benefits, including promotion of weight loss (Buemann *et al.*, 2000), no glycemic effect (Donner *et al.*, 1999; Seri *et al.*, 1993), anti-plaque, non-cariogenic, anti-halitosis, prebiotic, and anti-biofilm properties (Bertelsen *et al.*, 1999; Lærke *et al.*, 2000; Wong, 2000), enhancement of flavor (Rosenplenter and Mende, 2004), improvement of pregnancy and fetal development (Levin, 2000), treatment of obesity and reduction in symptoms associated with type 2 diabetes, hyperglycemia, anemia, and hemophilia (Levin 2002; Seri *et al.*, 1993). The heath benefits and applications of D-tagatose are summarized in Table 4.

Table 4. Health benefits and applications of D-tagatose (Oh, 2007).

Health benefit	**Applications**
Low caloric	Low carbohydrate diets, cereals, health bars, soft drink
No glycemic effect	Diabetic food (type 2)
Anti-halitosis	Anty-hyperglycemic agent, dietary supplement
Periodic	Chocolate, candy, chewing gum
Anti-biofilm antiplaque	Tooth paste, mouth wash
Flavor enhancement	Yogurt, bakery, milk-based drink, confectionery

D-Tagatose has attracted a great deal of attention in recent years due to its health benefits and similar properties to sucrose. Since it has been approved as GRAS (Table 5), D-Tagatose can be used as a low-calorie sweetener in non chronic drugs, tooth paste, mouth wash and in a wide variety of foods, beverages, health foods, and dietary supplements; it can be applied to products of low carbohydrate diets, cereals, health bars, chocolate, candy, chewing gum, yogurt, soft drink, bakery, milk-based drink, and confectionery. Its high solubility [58% (w/w) at 21°C] makes it ideal as a flavor enhancer or fiber in soft drinks and yogurts. D-Tagatose is also useful as an intermediate for synthesis of other optically active compounds and as an additive in detergent, cosmetic such as flavored lipstick, and pharmaceutical formulations (Ibrahim and Spradlin, 2000). Only 15% to 20% of D-tagatose ingested is absorbed from the small intestine to provide 1.5 kcal/g (Bertelsen *et al.*, 1999). The majority ingested is available for fermentation by colonic bacteria. The sugar D-tagatose has been shown through in vitro studies to stimulate colonic bacteria fermentation and production of short-chain fatty acids (eg, butyrate) and may have the potential to have prebiotic effects (Bertelsen *et al.*, 1999).

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Table 5. Main steps for the approval of the utilization of D-tagatose as food grade.

Date	Agency	Status	Reference
2001	USA Food and drug Administration (FDA)	GRAS (Generally Recognized as Safe)	FDA (2001)
July 2003	Korean Food and Drug Administration	Authorized the use of tagatose in foods	Bär (2004)
February 2004	Food Standards Australia New Zeland	Issued a favorable final assessment report permitting the use of tagatose as a novel food ingredient	FSANZ (2004)
June 2004	JECFA (Joint FAO/WHO Expert Committee on Food Addidives)	Stated that there is no need to limit the allowed daily intake of tagatose	INCHEM (2006)
December 2005	European Union (EU)	Tagatose was formally approved as a ‘novel food ingredient” without any restriction of usages	FSA (2005)

1.4.9 D-tagatose in bakery products

Bakery products may be formulated containing low levels of tagatose for its health benefits without altering the product's flavour. A study (Armstrong *et al.*, 2009) evaluated whether incorporating tagatose, into bakery products affected their flavours. Cinnamon muffins, lemon cookies, and chocolate cakes were prepared; 1 or 2% tagatose was added to the experimental products while an equal amount of sucrose was added to the control products. Untrained consumer panels were unable to distinguish between the flavour of products with and without tagatose. In addition, the flavours of bakery products containing and not containing tagatose were liked similarly.

Another study (Taylor *et al.*, 2008) investigated the suitability of tagatose, as a replacement for sucrose in cookies. A sucrose-containing cookie recipe was prepared as the control. Sucrose was replaced with tagatose at various levels ranging from 25% to 100%. The baked cookies were evaluated for spread, color, and hardness. When sucrose was replaced by tagatose, doughs with similar rheological properties to the control resulted. The tagatose-containing cookies were harder and darker with a lower spread than the control. Sensory data indicated that panelists liked the brown color of the 100% tagatose cookies better than the control, but disliked their sweetness. Overall likeness scores of the control and cookies made by replacing half of the sucrose with tagatose were the same. Tagatose appeared to be suitable as a partial replacer for sucrose in cookies based on similar dough properties, cookie properties, and likeness scores. Using tagatose to replace sucrose in foods would reduce the amount of metabolizeable sugars in the diet as well as provide the desirable prebiotic effect.

1.4.10 D-tagatose as antidiabetic and obesity control drug with several health benefits

D-tagatose has been suggested as a potential drug for type 2 diabetes thanks to its beneficial effects on postprandial hyperglycaemia and hyperinsulinaemia. In this paragraph the potential use of D-tagatose as drug as reported by Lu *et al.* (2008) is described.

Type II diabetes

Type 2 diabetes (regards 90% of the people with diabetes around the world) is generally characterized by two main abnormalities: peripheral insulin resistance and progressive failure of pancreatic β-cell function that leads to inadequate insulin secretion. The principal cause of diabetes complications (retinopathy, neuropathy, and arteriosclerosis) is hyperglycaemia caused by insufficient production of insulin and/or insulin resistance. Diet management, exercise and weight control may be not enough to achieve targeted glycaemic goals and many

individuals with type 2 diabetes ultimately require insulin as primary therapy with oral antidiabetic agents adjunctive therapy. However, glucose control in type 2 diabetics remains unsatisfactory because average concentration values of glycosylated haemoglobin (HbA_{1c}), a marker for average blood glucose levels over the previous months prior to the measurement, well above the normal range are reported in many epidemiologic studies.

D-tagatose as a drug for type II diabetes

Several studies showed that the pre-administration of D-tagatose reduces the rise in blood glucose after carbohydrate loading (glucose, sucrose or a controlled lunch). It seems that long-term glycaemic control by D-tagatose leads to a decline in HbA_{1c}. Spherix Incorporated (Tysons Corner, VA , USA) is currently conducting a phase 3 trial (NEET, Naturlose® Efficacy Evaluation Trial) to evaluate a placebo-subtracted treatment effect based on a decrease in HbA_{1c} levels. The study is a double-blind, placebo-controlled trial with patients randomized to receive 15 grams of D-tagatose three times daily with meals for a period of up to one year as an adjunct to diet and exercise, or a placebo three times daily with meals for up to one year as an adjunct to diet and exercise.

In October 2010 Spherix announced that preliminary results from the study showed a statistically significant ($p<0.05$) reduction in HbA_{1c} levels of 0.4% at 10 months in relatively healthy people with diabetes. The reduction was even more pronounced among patients treated in the U.S., and the reduction in HbA_{1c} generally increased over the 10 months patients were treated.

These findings are encouraging for the use of D-tagatose as a monotherapy for glycaemic control.

Weight control

For many diabetics, weight loss is the key to getting control of blood sugar, and may eliminate the need for medication. However, weight gain is the main side effect of insulin and drugs. D-tagatose does not increase insulin levels for glucose control. Instead, it blunts increase postprandial insulin. D-tagatose induces weight loss at medically acceptable rates, rendering the drug effective against obesity.

Effect on HDL levels

Dyslipidaemia is one of the factors associated with diabetes. It is also a major contributor to the increased coronary heart disease risk in patients with type 2 diabetes and is characterized by elevated levels of triglycerides and low-density lipoprotein (LDL) cholesterol and low

levels of high-density lipoprotein (HDL) cholesterol. Results of some clinical trials have demonstrated that treating patients who have low HDL with therapies that raise HDL can reduce major coronary events. In a 14-month study, HDL levels of patients taking D-tagatose rose from a mean baseline level of 30 to 41.7 mg/dl, thus it seems that D-tagatose increases HLD levels.

D-tagatose as a prebiotic

D-tagatose can be considered a prebiotic. A prebiotic is defined as 'a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that can improve host health'. Potential benefits of prebiotics are constipation relief, suppression of diarrhea and reduction of the risks of osteoporosis, atherosclerotic cardiovascular disease associated with dyslipideamia and insulin resistance, obesity and possibly type 2 diabetes. D-tagatose shows promise in this area. Studies on humans and animals have demonstrated that 20-25% of the amount of D-tagatose ingested is absorbed into the bloodstream passively, while the rest is fermented by intestinal microorganisms yielding short chain fatty acids ($SCFA_S$). In a human trial, D-tagatose ingestion of 10 g three times a day was also characterized by changes in microbial population species and densities. Pathogenic bacteria were reduced in numbers and beneficial bacteria were increased.

Plausible mechanisms for glycemic control

The mechanisms by which D-tagatose exerts its antihyperglycaemic effects are not entirely clear. Lu et al (2008) described a plausible mechanism of D-tagatose for glycemic control. Absorbed D-tagatose is metabolized mainly in the liver, following a metabolic pathway identical to that of fructose. D-tagatose is phosphorylated to D-tagatose-1-P by fructokinase. D-tagatose-1-P is split by aldolase to yield glyceraldehyde and dihydroxyacetone phosphate. Although aldolase acts on both fructose-1-P and D-tagatose-1-P, the cleavage of D-tagatose-1-P occurs at only about half the rate of the fructose-1-P resulting in a transient accumulation of D-tagatose-1-P that as fructose-1-P stimulates glucokinase activity, leading to an increased phosphorylation of glucose to glucose-6-P, which further activates glycogen synthase. It is possible that D-tagatose-1-P, similar to fructose-1-P, inhibits glycogen phosphorylase. The net effects of the regulation of these enzymes are to increase glycogen synthesis, and to decrease glycogen utilization (Fig. 5).

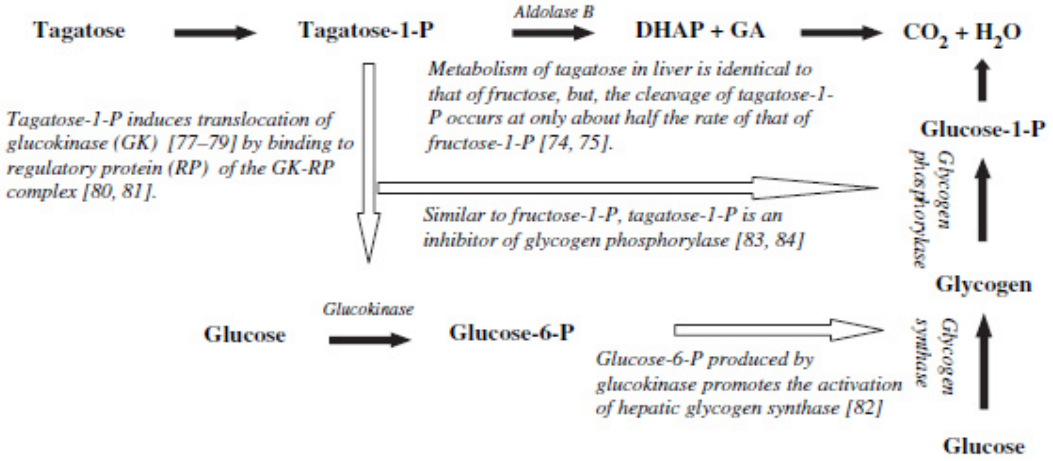

Figure 5. Plausible mechanism of actions for D-tagatose (from Lu *et al.*, 2008).

The present mechanisms suggest that D-tagatose exerts an anti-hyperglycaemic effect similar to that of fructose. However, at least in the short-term, fructose at high doses is associated with increase lipid synthesis, dyslipidaemia, deposition of lipid in the liver and skeletal muscle, insulin resistance, obesity and diabetic complications. Also fructose is a potent reducing sugar that promotes the formation of toxic advanced glycation and products. Because of fructose's adverse effect on plasma lipids, in 2002, the American Diabetes Association recommended avoiding fructose other than what occurs naturally in fruits.

In addition to the effect on glycogen regulation, D-tagatose inhibits sucrose, leading to the suppression of sucrose digestion in the small intestine. An in vitro study found that D-tagatose also inhibits the activity of maltase derived from rabbit small intestine mucous membrane and thereby delays the digestion of starch.

In summary, the overall effect of D-tagatose, probably together with other unknown mechanisms, is to depress the rise in blood sugar by increasing glycogen synthesis, decreasing glycogen utilization and possibly also by reducing the digestion of sucrose and other carbohydrates in the small intestine.

2. Methods for the production of rare sugars

The basis for the production methods of monosaccharides was laid by extensive research on organic chemistry by E. Fisher in the later half of the 1800s. The structures of monosaccharides were determined in this era, mostly with the establishment of their production methods. For this reason, no systematic research was conducted on the synthesis of monosaccharides, which was regarded as having been completed. It was also hampered by the great difficulty in the synthesis of monosaccharides by techniques of organic chemistry because of their complex chiral structures. In recognition of the central role that carbohydrates play in many biological processes, there has been enormous effort by organic chemists to make unnatural and rare sugars; none of these attempts has allowed the preparation of substantial quantities of material and involve the use of many steps by environmentally unfriendly procedures.

2.1 Bioproduction strategies for rare sugars

It is advantageous to use cheap feedstocks, such as starch, wood or whey, as starting materials for the production of rare sugars. D-Glucose is obtained from starch whereas D-xylose can be derived from the hemicellulosic fraction of wood. Whey contains lactose, which can be hydrolysed into D-glucose and D-galactose. Izumori (2006), devised a scheme to display all the rare sugars in a ring-form called an “Izumoring”. Using this display, it is possible to describe the biochemical reactions needed to produce them and the connection between the D- and L-configurations of these sugars. Three different Izumorings have been proposed, one for

tetroses, one for pentoses, and one for hexoses. Using this approach, it has been possible to produce many rare sugars allowing the determination of their physical (such as conformation, solution structure and solubility), chemical and biological properties. However, there is still much to be done to determine the best biocatalytic routes for the production of specific rare sugars to ultimately produce them in optimal bioprocessing scheme.

Figure 6 shows the interconversion of all 3-carbon, 5-carbon and 6-carbon sugars by Izumoring. The red circles are aldoses, blue circles are ketoses, and yellow circles are polyols. Hexoses, as well as pentoses or trioses are connected to one another with a line, which represents enzyme reactions. The red lines are aldose isomerase (catabolic reaction between aldose and ketose), blue lines are polyol ketose oxidoreductase (enzymes that oxidize or reduce carbon 2 to transform polyols to ketoses), yellow lines are D-tagatose-3-epimerase (an enzyme that epimerizes position 3 of all free ketohexoses), and white lines are aldose reductase (enzymes that reduce aldoses to the corresponding polyols). All D-type sugars are shown in the blue region in the right, all L-type sugars in the red region in the left, and polyols and meso-polyols in the yellow region in the middle. In order to show how the display can be used, we can follow the route of the Izumoring from D-fructose to L-fructose for the production of L-fructose via four reactions. The first step is epimerization of D-fructose to D-psicose by D-tagatose 3-epimerase. The second step is reduction of D-psicose to allitol by oxidoreductase. The third reaction is transformation of allitol to L-psicose by oxidoreductase. The final step is epimerization of L-psicose to L-fructose by D-tagatose 3-epimerase.

In the following paragraphs aldose isomerases and tagatose epimerases will be described in detail.

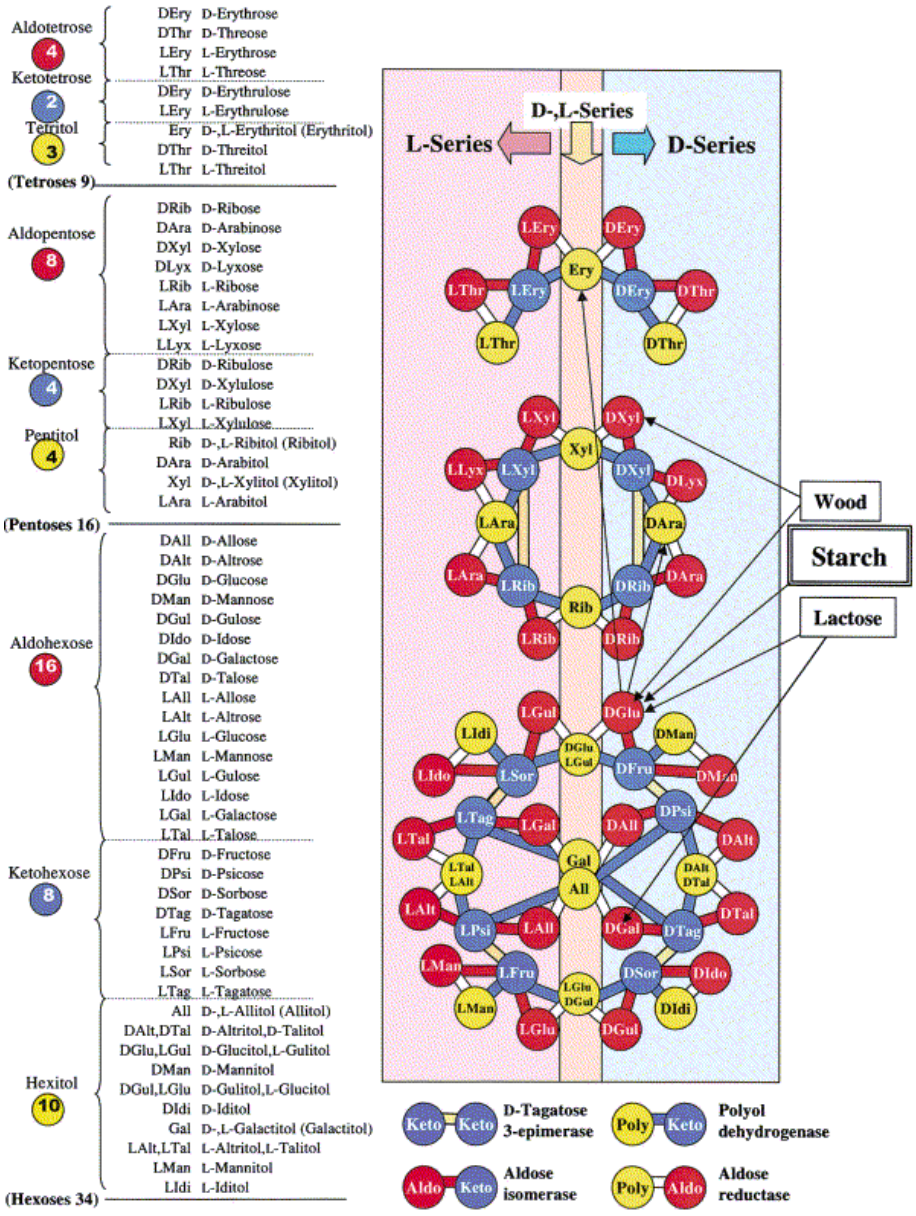

Figure 6. Izumoring strategy to design the production processes of all monosaccharides from readily available raw materials; starch, wood and lactose (from Granström *et al.*, 2004).

2.2 Aldose isomerases and tagatose epimerases

2.2.1 D-xylose isomerase

The chemical conversion of glucose to fructose, carried out at high pH and temperature (Barker *et al.*, 1973) is non-specific and leads to the formation of undesirable colored

products, the formation of off-flavors, and sweetness reduction. For these reasons, commercial HFCS production has since the 1960s employed an enzymatic conversion using xylose isomerase (EC 5.3.1.5) (XI). The advantages are milder pH and temperature conditions, no formation of side products, and potential for catalyst improvement via molecular evolution. XI mediates "isomerization" between an aldose and a ketose. In XI, it occurs through a "hydride shift" mechanism (ring opening of substrate, isomerization via a hydride shift from C-2 to C-1, ring closure of product), and a divalent cation (i.e., Mg^{2+}, Mn^{2+}, Co^{2+}) plays an important role in the mechanism (Kim, 2004). Several studies have focused on improving XI activity on glucose, through site directed mutagenesis, to create a true "glucose isomerase" [Meng *et al.*, 1991; Sriprapundh *et al.*, 2003). Site-directed mutagenesis and molecular evolution provided excellent tools for the alteration of optimal pH in XIs (Sriprapundh *et al.*, 2003; van Tilbeurgh *et al.*, 1992).

XIs have been reported to catalyze several different rare sugar reactions. The use of divalent ions (i.e., Mg^{2+}, Co^{2+} and Mn^{2+}) is required for activity in most XIs. Pastinen *et al.* (1999) found that D-xylose isomerase can be used in the production of various rare aldohexoses from ketohexoses, such as D-altrose, and D-allose from D-psicose, as well as D-gulose and D-idose from D-sorbose. The use of Co^{2+}, in particular, often leads to optimal catalytic efficiency. However, due to potential toxicity of this cation in foods and pharmaceuticals, investigations of XIs for rare sugar synthesis have focused on the Mg^{2+}-dependent, commercially available *Streptomyces* XIs.

Although efforts have been made to improve enzyme/substrate ratios (e.g., using cross-linked *Streptomyces rubiginosus* XI crystals in bioreactors and effluent recycling), yields in rare sugar reactions remain extremely low (Jokela *et al.*, 2002). Rare sugars are expensive to produce, especially when including a chromatography step is needed to remove a toxic metal. Highly thermostable XIs from hyperthermophiles, such as *Thermotoga maritima* (Brown *et al.*, 1993) and *T. neapolitana* (Hess *et al.*, 1998; Sriprapundh *et al.*, 2003), are candidates for rare sugar production, especially if protein-engineering techniques can improve their efficacy in this regard.

2.2.2 L-Arabinose isomerase

L-arabinose isomerase (EC 5.3.1.4) (AI) catalyzes the conversion of D-galactose to D-tagatose (Figure 7) as well as the conversion of L-arabinose to L-ribulose, owing to the similar configurations of the substrates (Cheetham and Wootton, 1993; Roh *et al.*, 2000).

Many AI genes have been cloned from various bacterial species: *E. coli* strains, *Salmonella typhi*, *S. typhimurium*, *Yersinia pestis*, *Shigella flexneri*, *Vibrio parahaemolyticus*, *Bacillus subtilis*, *B. halodurans*, *Oceanobacillus iheyensis*, *Lactobacillus plantarum*, *Clostridium acetobutylicum*, *Bifidobacterium longum*, *Bacteroides thetaiotaomicron*, *T. maritima*. These AIs are all potential candidates for galactose isomerization. Since the process of D-tagatose isomerization from D-galactose is commercially viable, much of the relevant information on AI has been recently documented in patents (Kim, 2004). Among other patented sources, the AIs of *G. stearothermophilus* (Kim *et al.* 2001a), *Thermotoga neapolitana* (Kim *et al.*, 2002), *Thermus sp.* (Kim *et al.* 2003b) and *Thermoanaerobacter mathranii* (Jørgensen *et al.*, 2004) have been reported as D-tagatose isomerization enzymes.

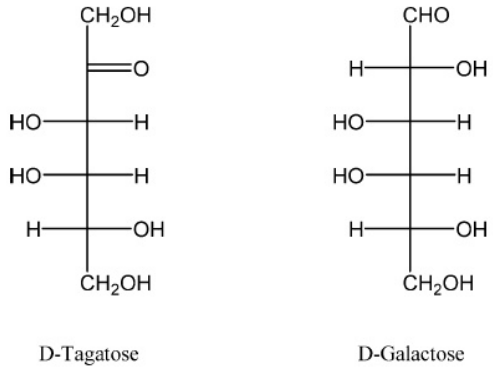

Figure 7. Chemical structures of D-tagatose and D-galactose.

The structure of AI has not yet been solved. To understand the reaction mechanism, X-ray crystallography studies are required. Only a few AI have been purified and characterized. Kim reported that *E. coli* AI is composed of six identical subunits and has a molecular weight of approximately 60000 Dalton (Kim, 2004). *E. coli* AI is activated by Mn^{2+}, implying that divalent cations play an important role in the enzyme reaction mechanism. The AIs from *Lactobacillus gayonii* and *Aerobacter aerogenes* are also activated by divalent cations (Kim, 2004). The kinetic parameters of various bacterial L-arabinose isomerases for D-galactose isomerization are presented in Table 6.

Table 6. Kinetic parameter of L-arabinose isomerase (Oh, 2007).

Strain for enzyme source	**V_{max} (U/mg)**	**K_m (mM)**	**k_{cat} (min^{-1})**	**k_{cat}/K_m (-)**
Aerobacter aerogenes US100	NR	270	NR	NR
Alicyclobacillus acidocaldarius	7.5	129	NR	NR
Bacillus halodurans	1.3	167	120	0.4
B. stearothermophilus	8.9	57	438	8.5
Escherichia coli	NR	1480	NR	NR
Geobacillus stearothermophilus T6	9	120	516	4.3
G. stearothermophilus	7.8	145	173	1.2
G. stearothermophilus (mutant enzyme)	37.6	578	1.211	2.1
G. thermonitrificans	6.9	408	204	0.5
G. thermonitrificans (mutant enzyme)	35.4	399	1046	3.1
Thermoanaerobacter mathranii	NR	120	NR	NR
Thermotoga neopolitana	14.3	250	810	3.2
Thermotoga maritima	8.9	60	504	8.4

A summary of D-tagatose production from D-galactose by various L-arabinose isomerases and cells containing the enzyme is shown in Table 7. The D-tagatose equilibrium conversion from D-galactose depends on the isomerization temperature and it is shifted toward D-tagatose at higher temperatures (Oh, 2007): 28.8% at 30°C in *E. coli* L-arabinose isomerase (Kim *et al.*, 2001b), 58% at 65°C in the enzyme from *G. thermodenitrificans* (Oh *et al.*, 2006) and 68% at 85°C in *Thermotoga neapolitana* (Kim *et al.* 2002). Figure 8, reports a plot of the maximum final ratio of D-tagatose and D-galactose, which was assumed as the equilibrium ratio, as a function of isomerization temperature (data extracted from Table 7).

Table 7. D-tagatose production from D-galactose by L-arabinose isomerases and cells containing the enzyme: enzyme form (E, free enzyme; IE, immobilized enzyme; I, immobilized cells), T (temperature), D-gal (initial D-galactose concentration), D-tag (final D-tagatose concentration), X (D-galactose conversion), P (productivity). Adapted from Oh (2007).

Form	Strain for enzyme source	T (°C)	D-gal. ($g\ l^{-1}$)	D-tag. ($g\ l^{-1}$)	X (%)	P ($g\ l^{-1}\ d^{-1}$)
E	*Bacillus stearothermophilus*	70	0.9	0.4	48.0	1.5
E	*Escherichia coli*	30	100	29	28.8	4.1
IE	*Escherichia coli*	30	500	103	20.5	7.5
IE	*Escherichia coli*	37	100	30	30.0	30
E	*Geobacillus stearothermophilus*	60	100	31	30.6	46
IE	*Geobacillus stearothermophilus*					
	(batch)	60	500	230	46.0	319
	(continuous)	60	300	145	48.3	1296
I *E. coli*	*Geobacillus stearothermophilus*	60	300	59	19.7	70
E	*Geobacillus thermodenitrificans*	60	300	158	52.7	190
E	*Termoaraerobacter mathranii*	65	300	126	42.0	63
E	*Thermotoga neapolitana*	80	1.8	1.2	68.0	1.5
I *E. coli*	*Thermotoga neapolitana*	70	180	49	27.2	98
IE	*Thermotoga neapolitana*	70	300	138	46	20.7
E	*Thermotoga mathranii*	70	1.8	1.0	56.0	4.0
E	*Thermos sp.*	60	1.0	0.5	54.0	0.2

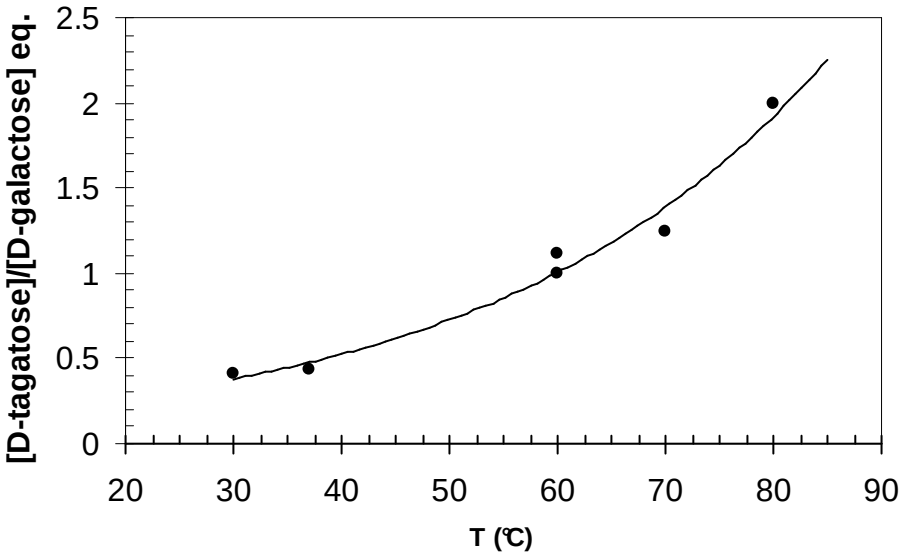

Figure 8. Effect of temperature on the equilibrium ratio of D-tagatose and D-galactose concentrations.

The highest levels of D-tagatose production have been reported as 230 g l^{-1} D-tagatose from 500 g l^{-1} D-galactose using immobilized *G. stearothermophilus* L-arabinose isomerase in continuous recycling mode of a packed-bed bioreactor with a tagatose productivity of 319 g l^{-1} day^{-1} (Kim *et al.* 2003a). In continuous mode using the immobilized enzyme, 145 g l^{-1} D-tagatose from 300 g l^{-1} D-galactose was produced in the bioreactor with an average tagatose productivity of 1296 g l^{-1} day^{-1} as the reported highest productivity (Ryu *et al.*, 2003), which was 5.6-fold higher than that in continuous recycling mode. The highest reported yield of D-tagatose, which is catalyzed by *Thermotoga neapolitana* L-arabinose isomerase using 1.8 g l^{-1} D-galactose, is 68% at 85°C (Kim *et al.*, 2002).

2.2.3 L-Rhamnose Isomerase

L-rhamnose isomerase (EC 5.3.1.14) reversibly isomerizes L-rhamnose to its corresponding ketose, L-rhamnulose.

An L-rhamnose isomerase from a mutant strain of *Pseudomonas aeruginosa* (sp.) strain LL172 showed a broad specificity to various rare aldoses and ketoses. The highest activity was detected against L-rhamnose (100%), followed by L-lyxose (97%). However, this enzyme was active on L-mannose, D-gulose, D-ribose, D-allose, and L-talose. This L-rhamnose isomerase was also immobilized on chitopearl beads and produced L-mannose from L-fructose at 60^{o}C, with a 30% yield (Bhuiyan *et al.*, 1997).

A L-rhamnose isomerase from *Pseudomonas stutzeri* (Leang *et al.*, 2004) had a broad specificity being active on L-mannose, L-lyxose, L-glucose, L-tagatose, D-allose, and D-ribose.

Large-scale production of D-allose from D-psicose using a continuous bioreactor containing immobilized L-rhamnose isomerase from *P. stutzeri* has also been reported (Morimoto *et al.*, 2006). A 10% conversion rate of D-psicose to D-allose was achieved, following the completion of a 17-day reaction period.

2.2.4 L-Fucose Isomerase

L-Fucose Isomerase (FI), isolated from *E. coli* catalyzes reversible ketol isomerization between sugars with stereo-specific C2 and C3 centers (Fessner *et al.*, 2000). FI, a homohexamer, required divalent ions (Mn^{2+} and Co^{2+}) for activity. D-tagatose-3-epimerase from *Pseudomonas cichorii* ST-24 was combined with L-fucose isomerase from *Klebsiella pneumoniae* 40bXX to produce D-altrose from D-fructose at an overall yield of 6% (Menavuvu *et al.*, 2006). This work was significant as a proof of concept of producing D-altrose from an inexpensive substrate, D-fructose.

2.2.5 D-tagatose-3-epimerase

D-tagatose-3-epimerases from *Pseudomonas cichorii* ST-24 (Ishida *et al.*, 1997; Takeshita *et al.*, 2000) and *Pseudomonas* sp. (Itoh *et al.*, 1995) have been characterized. Further efforts on D-tagatose-3-epimerase have focused on the conversion of L-sorbose and L-psicose to L-tagatose and L-fructose, respectively (Itoh and Izumoring, 1996), D-fructose to D-psicose (Itoh *et al.*, 1995), and D-tagatose to D-sorbose (Itoh *et al.*, 1995). Immobilized D-tagatose-3-epimerase (chito pearl beads) produced D-psicose from D-fructose with a yield of 25%, compared to the unbound form of D-tagatose-3-epimerase that yielded 20%. This immobilized form of D-tagatose-3-epimerase also produced D-sorbose from D-tagatose with a yield of 70%. D-tagatose-3-epimerase from *Rhodobacter sphaeroides* converted D-fructose to D-psicose (17% conversion). This study compared the relative activities of the *P. cichorii* D-tagatose-3-epimerase and *Agrobacterium tumefaciens* D-psicose-3-epimerase with the D-tagatose-3-epimerase from *Rhodobacter sphaeroides* and showed the *R. sphaeroides* enzyme was more active on D-fructose at 40^{o}C; all of these enzymes required the metal Mn^{2+} for activity. In addition, *A. tumefaciens* D-psicose-3-epimerase produced D-psicose from D-fructose with a yield of 99%.

2.3 Critical aspects in the biological production of D-tagatose

In the future, L-arabinose isomerase will be able to produce industrially D-tagatose because the biotransformation has some advantages over the chemical process, such as mild reaction conditions, D-tagatose production without by-products, no salt in waste water, and D-galactose recycling during purification (Oh, 2007).

The biological production of D-tagatose has been studied using several substrates: galactitol, D-sorbose, D-psicose and D-galactose.

2.3.1 Galactitol to D-tagatose

The Izumori group firstly reported in 1984 that tagatose was produced from several microorganisms by oxidation of galactitol. *Arthrobacter globiformis*, *Gluconobacter oxydans*, *Mycobacterium smegmatis*, *Enterobacter agglomerans* and *Klebsiella pneumoniae* have been reported to convert galactitol into D-tagatose (Oh, 2007). The responsible enzyme for this biotransformation is a sorbitol dehydrogenase (Rollini and Manzoni, 2005). Since the cost for galactitol as a raw material is relatively high (US $500–800/kg), this process is not considered for bulk scale tagatose production (Kim, 2004).

2.3.2 D-sorbose to D-tagatose

Agrobacterium tumefaciens D-psicose 3-epimerase (Kim *et al.* 2006a) and *Pseudomonas cichorii* D-tagatose 3-epimerase convert D-sorbose to D-tagatose (Itoh *et al.*, 1994; Ishida *et al.*, 1997; Yoshida *et al.*, 2007). However, D-sorbose is an expensive substrate that seems to have little potential for commercial application.

2.3.3 D-psicose to D-tagatose

Various strains of *Mucoraceae* fungi convert D-psicose to D-tagatose (Yoshihara *et al.*, 2006). As the mass production of D-psicose from D-fructose has become industrially feasible in recent years (Kim *et al.*, 2006a; Takeshita *et al.*, 2000), the production of D-tagatose from D-fructose via D-psicose as an alternative method has been proposed.

2.4 D-galactose to D-tagatose

To overcome the disadvantages of the chemical isomerization of D-galactose to D-tagatose, biological manufactures of D-tagatose using several biocatalyst sources have been studied intensively in recent years.

The cloned L-arabinose isomerizes of *Escherichia coli*, *Bacillus subtilis*, and *Salmonella typhimurium* mediate the conversion of D-tagatose from D-galactose (Roh *et al.* 2000). L-Arabinose isomerase has been of interest and studied intensively in recent years due to its industrial feasibility in D-tagatose production (Kim *et al.* 2003a). Details about this isomerization are reported above.

For effective D-tagatose productions using L-arabinose isomerase, the reaction conditions such as pH, temperature, and metal ion should be optimized. The optimal reaction conditions of these bacterial L-arabinose isomerases are compared in Table 8 (Oh, 2007).

Table 8. Optimal reaction conditions of L-arabinose isomerase for the isomerisation of D-galactose to D-tagatose.

Strain for enzyme source	**Optimum T (°C)**	**Optimum pH**	**Half-life (min)**	**Metal ion**
Aerobactere aerogenes	50	6.4-6.9	NR	Mn^{2+}
Alicyclobacillus acidoca Idariuse	65	6	NR	Mn^{2+}
Bacillus alodurans	50	7.5-8.0	20 (70 °C)	Mn^{2+}
B. stearothermophilus US100	80	7.5-8.0	110 (75 °C)	NR
Escherichia coli	30	8.0	60 (50 °C)	Fe^{2+},Mn^{2+}
Geobacillus stearothermophilus T6	70	7.0-7.5	52 (80 °C)	Mn^{2+}
G. stearothermophilus	65	7.5	17 (70 °C)	Mn^{2+}
G.theromonitrificans	70	8.5	42 (75 °C)	Mn^{2+}
Lactobacillus gayonii	30-40	6.0-7.0	NR	Mn^{2+}
Mycobacterium sgmegamtis	45	7.0-7.5	10 (45 °C)	Mn^{2+}
Thermoanaerobacter mathranii	65	8.0	NR	Mn^{2+}
Termotoga neopolitana	85	7.0	120 (90 °C)	Co^{2+}
T. maritima	90	7.0-7.5	185 (90 °C)	Co^{2+},Mn^{2+}
Thermus sp.	60	8.5	NR	Mn^{2+}

2.4.1 pH

The optimum pH of enzymatic D-galactose isomerization ranges from pH 6.0 to 8.5. The optimum pH for L-arabinose isomerase from *Alicyclobacillus acidocaldarius* is 6.0 (Lee *et al.* 2005), while the enzymes from *G. thermodenitrificans* and *Thermus sp*. have optimum pH of 8.5 (Kim and Oh 2005; Kim *et al.* 2003b). The optimum pHs of the others are in the range 6.0–8.0. Commercial application of AI requires an acidic pH range to reduce non-specific side reactions.

2.4.2 Temperature

Poor thermal stability of AI would limit the operation time of the reactor because industrial tagatose production is suggested to be carried out at 60–65°C in an immobilized enzyme reactor (Kim *et al.*, 2003a; Oh *et al.*, 2001), because the galactose-tagatose equilibrium shifts toward tagatose at higher temperatures (See Figure 8). Operation at temperatures higher than 80°C, however, would cause a browning reaction as well as isomerization. The optimum temperature for L-arabinose isomerase (Table 8) is in the range of 30–50°C in mesophiles such as *Aerobacter aerogenes*, *Alicyclobacillus acidocaldarius*, *Bacillus halodurans*, *Escherichia coli*, *Lactobacillus gayonii* and *M. smegmatis*; of 60–80°C in thermophiles such as *B. stearothermophilus*, *Geobacillus stearothermophilus*, *G. thermodenitrificans*, *Thermus sp.*, and *Thermoanaerobacter mathranii*; and of 85-90°C in hyperthermophiles such as *Thermotoga neapolitana* and *T. maritima* (Oh, 2007).

2.4.3 Metal ion

The mesophilic and thermophilic L-arabinose isomerases require Mn^{2+} as a cofactor to enhance the isomerization reaction rate, while the hyperthermophilic L-arabinose isomerases require Co^{2+} (Kim *et al.* 2002; Lee *et al.* 2004) or Mn^{2+}.

The use of Co^{2+}, in particular, often leads to optimal catalytic efficiency. However, due to potential toxicity of this cation in foods and pharmaceuticals their use in commercial tagatose production might not be approved in the food industry.

2.4.4 Specificity

All reported bacterial L-arabinose isomerases have higher specificity for L-arabinose than D-galactose. The ratio of catalytic efficiency (k_{cat}/K_m) for D-galactose to L-arabinose decreased when the optimum temperature of L-arabinose isomerase was increased; the ratio of kcat/Km was 128 in the mesophilic enzyme from *B. halodurans*, 51 in the thermophilic enzyme from

G. stearothermophilus, respectively, and 9 in the hyperthermophilic enzyme from *T. maritima*.

These results suggested that substrate specificity at high temperatures altered from L-arabinose into D-galactose. The specificity of AI for D- galactose should be increased.

2.4.5 Equilibrium-shifted production of tagatose

The equilibrium between substrate and product cannot be simply changed by protein engineering of the enzyme, as it is controlled by the reaction temperature (Chang *et al.* 1999). To obtain a higher conversion yield of product than that defined at the specific temperature, the equilibrium should be shifted toward product. If a compound (like boric acid) binds more selectively to product (D-tagatose) than substrate (D-galactose), an equilibrium shift occurs. The formation of the product–compound complex has been proposed as an alternative to high-temperature biotransformation for feasible industrial production of D-tagatose.

2.4.6 L-arabinose isomerase protein engineering

For the industrial use of AI for D-tagatose production, a few characteristics of this enzyme should be altered. Recent developments in DNA techniques and protein engineering tools (i.e., directed evolution, high throughput screening, site directed mutagenesis, 3D structure analysis) provide encouraging possibilities for developing the desirable enzyme characteristics. Mutations on or near the active site of the enzyme could change the substrate specificity and alter the pH range for activity.

Based on the solved crystal structure and sequence alignments, the residues for the essential catalytic site and substrate recognition of *G. stearothermophilus* US 100 L-arabinose isomerase was suggested (Rhimi *et al.* 2007).

Directed evolution of the L-arabinose isomerase gene has been suggested as a powerful tool for increasing the reaction rate (Kim *et al.* 2001a). A mutated *G. stearothermophilus* L-arabinose isomerase exhibiting the change of three amino acids compared to the wild-type enzyme was isolated. The mutated enzyme showed increases in D-galactose isomerization activity, optimum temperature, catalytic efficiency (kcat/Km) for D galactose, and the production rate of D-tagatose from D galactose (Kim *et al.* 2006b). A double-site mutant enzyme of L-arabinose isomerase from *G. thermodenitrificans*, was obtained by site-directed mutagenesis. The double-site mutant enzyme converted D-galactose to D-tagatose with a yield of 58%, whereas a wild type gave 46% of D-tagatose conversion after 300 min at 65°C (Oh *et al.* 2006).

2.4.7 D-tagatose production using immobilized biocatalyst

To produce D-tagatose effectively, L-arabinose isomerase or cells containing the enzyme should be immobilized. Immobilized *G. stearothermophilus* L-arabinose isomerase entrapped by alginate produces D- tagatose at an average D-tagatose productivity of 1296 g l^{-1} day^{-1} as the reported highest productivity (Ryu *et al.*, 2003).

The immobilized *E coli* cells containing *G. stearothermophilus* L-arabinose isomerase in alginate produces a productivity of 70 g l^{-1} d^{-1} in a bioreactor (Jung, *et al.* 2005) while the immobilized *E coli* cells containing *Thermotoga neapolitana* L-arabinose isomerase attained a productivity of 98 g l^{-1} day^{-1} (Hong *et al.*, 2007).

2.4.8 GRAS host

D-Tagatose production by enzyme or cell of generally recognized as safe (GRAS) host should be studied for the use of tagatose as food to avoid any potential poisoning problems (Burdock and Carabin, 2004). The problem can be solved by transferring the gene of L-arabinose isomerase to GRAS host such as *Bacillus megaterium*, *Corynebacterium ammonagenes*, and *C. glutamicum*. Efforts to develop industrially feasible tagatose production using AI are underway.

2.5 Chemical production of D-tagatose

D-tagatose is commercially produced by isomerization of D-galactose, which is prepared from lactose. The conversion of D-galactose into D-tagatose can occur in one of two ways: using a chemical process (Beadle *et al.*, 1992) or an enzymatic process (Kim *et al.*, 2003a) (Fig. 9). The current production process, based on the chemical isomerization was developed and recently patented by Spherix Incorporated (Tysons Corner, VA , USA) (Beadle *et al.*, 1991; Beadle *et al.*, 1992).

In the chemical process D-galactose is isomerized to D-tagatose under alkaline conditions preferably using calcium hydroxide as a complexant. Calcium hydroxide shifts the isomerization equilibrium between D-galactose and D-tagatose in the direction of D-tagatose because it forms an insoluble complex with this sugar at elevated pH (pH 12). In the precipitated calcium complex, D-tagatose is protected and does not participate in side-reactions that usually occur with sugars under the applied alkaline conditions. Treatment of the suspension with carbon dioxide liberated D-tagatose by neutralizing the mixture and by precipitating calcium as calcium carbonate (Beadle *et al.*, 1992) (Fig. 9).

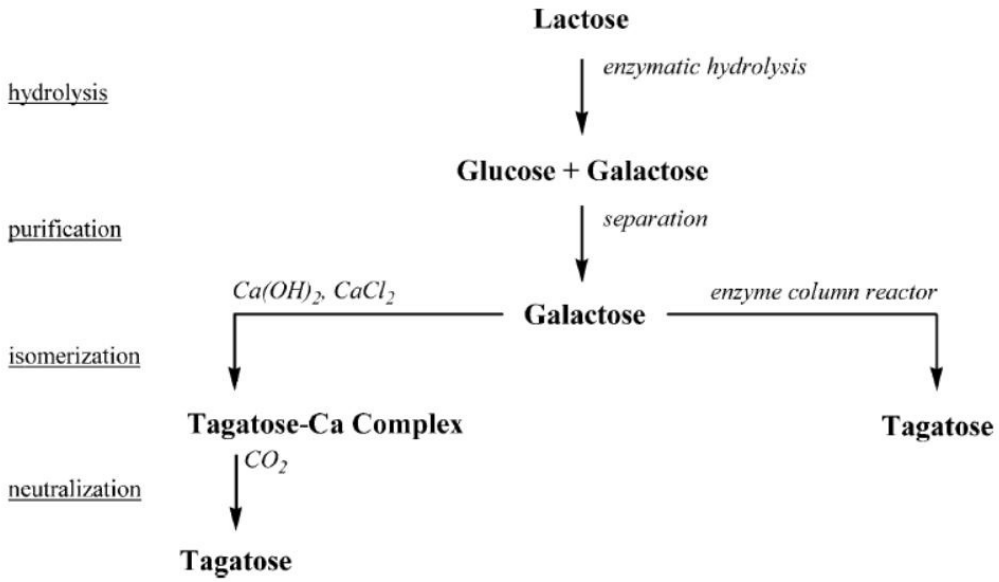

Figure 9. Schematic enzymatic and chemical process of D-tagatose production. The enzymatic isomerization process is described in detail in chapter 2, and involves the use of a thermostable L-arabinose isomerase.

3. Immobilized-enzyme technology

Biocatalysts are able to catalyse reactions under mild conditions of pH, temperature and pressure with high efficiency, specificity and selectivity. Among the techniques available to improve the enzyme features, immobilization has been revealed in the last times as a very powerful tool, if properly designed. Since 1972, when Chibata and coworkers pioneered the use of immobilised enzymes in industrial processes, numerous methods of immobilisation on a variety of different materials have been developed in order to improve the stability, activity and reusability of enzymes and whole cells. Numerous books and reviews on several specific aspects of immobilized enzymes are available today. The reader is addressed to Blanch and Clark (1996), Bickerstaff (1997), Chibata *et al.* (1986) and Hartmeier (1985) for a general overview on the subject.

3.1 Advantages and limitations of immobilized enzymes

Table 1 summarises advantages and disadvantages of immobilized enzymes and cells.

Table 1. Advantages and disadvantages of enzyme and whole cell immobilization (adapted from van de Velde *et al.*, 2002).

Advantages	Disadvantages
Easy recycling of catalyst	Extra costs
More simple down-stream processing	Loss of activity
Stabilization of enzymes	Diffusion limitations
Good reaction control	Increased catalyst volume
Flexibility of reactor choice (continuous of fixed bed)	A time-consuming effort to obtain a suitable carrier

The industrial-scale use of a relatively costly enzyme as a catalyst generally asks for its recovery and reuse for long times to make a process economically feasible. Moreover, the use of an immobilized enzyme allows to simplify the design of the reactor and the control of the reaction (Bickerstaff, 1997; Chibata *et al.*, 1986; Hartmeier, 1985; Katchalski-Katzir, 1993; Cheetham, 1993). However, the stability of the enzyme should allow its reuse for long times. Therefore, the enzyme needs to be very stable or to become highly stabilized during the immobilization process. Finally, since the final use of the enzyme derivative will be as industrial catalyst, the ideal immobilization processes should limit the use of toxic or unstable reagents, be very simple, robust, etc.

3.2 Immobilisation techniques

The available methods of immobilization can be divided into five different categories, i.e., adsorption, ionic binding, covalent attachment, cross-linking, and entrapment. The following section gives an overview of the different methods as extracted from van de Velde *et al.* (2002). Figure 1 depicts the different methods while Table 2 reports the characteristics of the different methods of enzyme immobilization and corresponding examples with supports and reagents used for each method.

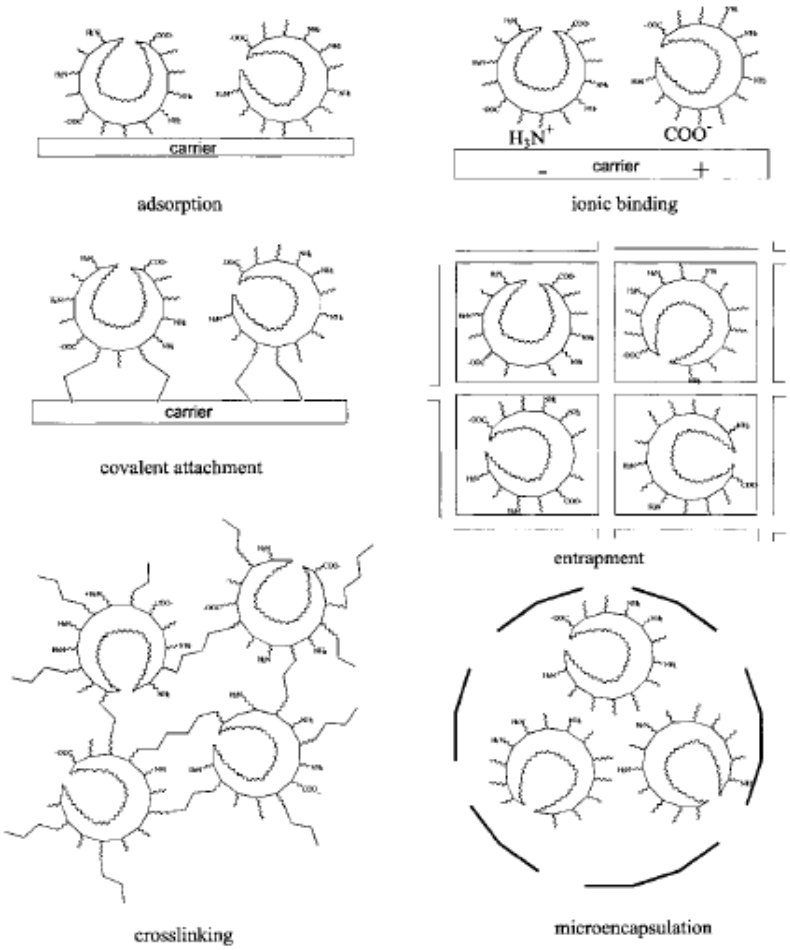

Figure 1. Schematic representation of the main enzyme immobilisation techniques (from van de Velde *et al.*, 2002).

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3.2.1 Adsorption

Adsorption of enzymes is based on physical interactions between the biocatalyst and the support surface. The bond is generally weak, non-specific and affected by pH, temperature, and contact with salts and solvents. In most of the cases, the support is macroporous and adsorbs the enzyme at the internal pore surface. Immobilized derivatives obtained by adsorption can be subject to leaching of the enzyme to the aqueous medium. The use of cross-linking after adsorption may prevent leaching and increase enzyme stability, although activity can be adversely affected (Woudenberg -Van *et al.*, 1998).

3.2.2 Ionic Binding

In ionic binding procedures, enzymes are regarded as polyelectrolytes that will bind to supports of opposite charge. Ionic binding of enzymes gives much more stable preparations than physical adsorption, although the stability of the preparation may be sensitive to changes in pH and ionic strength. Hence, the carrier is easy to regenerate. Whether the carrier has an anionic or a cationic character, the resulting enzyme load and activity will depend on the enzyme charge.

3.2.3 Covalent Attachment

Covalent attachment is probably the most widely used method of enzyme immobilization. The bond is created through the reaction of reactive groups at the protein surface, e.g., the N-terminus, lysine amino groups or hydroxy, sulphydryl, or phenolic functional groups, with corresponding groups at the surface of water-insoluble supports. Examples of carriers which are used for the covalent immobilisation of enzymes are activated cellulose, dextran and starch (pre-activated via the formation of reactive imidocarbonates from native hydroxy groups and cyanogen bromide), isocyanate prepolymers and the very popular Eupergit C or C250L (copolymer of methylacrylamide containing oxirane groups).

One of the advantages of covalent attachment of enzymes compared to other immobilisation methods is the resulting increased stability. However, the stronger interaction with the carrier decreases the enzyme molecular flexibility, which may have a negative effect on activity. Finally, the often expensive carrier material generally cannot be reused.

3.2.4 Cross-Linking

Cross-linking involves the connection of molecules of enzyme to each other via covalent bonds. They usually react with amino groups at the outer surface of the protein. The method is very simple and allows to obtain an almost pure immobilised enzyme, but the process is difficult to control. Crosslinking of protein precipitates can be more easily controlled. Robust catalysts have been obtained via the cross-linking of enzyme crystals and, recently, good results were obtained with so-called cross-linked enzyme aggregates. Although cross-linked enzyme crystals are chemically and mechanically quite robust catalysts, the development of suitable protocols for crystallisation and cross-linking is time-consuming and labour-

intensive, which is a distinct disadvantage of these preparations, when compared with cross-linked enzyme aggregates.

Table 2. Comparisons of the characteristics of different methods of enzyme immobilization and corresponding examples with supports and reagents used. Table adapted from Bailey and Ollis (1986) and van de Velde *et al.* (2002).

	Carrier binding method					
Characteristic	**Adsorption**	**Ionic binding**	**Covalent binding**	**Cross-linking**	**Entrapping**	**Micro-encapsulation**
Preparation	Easy	Easy	Difficult	Difficult	Difficult	Difficult
Activity	Low	High	High	Moderate	High	-
Substrate specificity	Unchangeable	Unchangeable	Changeable	Changeable	Unchangeable	Unchangeable
Binding force	Weak	Moderate	Strong	Strong	Strong	Strong
Regeneration	Possible	Possible	Impossible	Impossible	Impossible	Impossible
General applicability	Low	Moderate	Moderate	Low	High	-
Cost of immobilization	Low	Low	High	Moderate	Low	-
Support, Reagents (R)	Accurel EP100 Mesoporous molecular sieves Silica Eudragit S-100 Teflon	DEAE-cellulose Chitosan beads Amberlite DEAE-Sephadex Cellulose phosphate Dextran sulphate	Cellulose Dextran Starch Isocyanate prepolymers Eupergit C Eurpergit C250L	Glutaraldehyde (R) Diazobenzidine (R) Tannic acid (R) Dimethyl adipimidate (R)	Polyacrylamides	Nylon Carrageenan Cellulose nitrate 1,6-diaminohexane

3.2.5 Entrapment

Entrapment is based on the encapsulation of enzymes within matrices, membranes, or lattice structures without any modification or binding of the enzyme. Entrapment in a polymeric matrix can be accomplished by polymerising or cross-linking, e.g., thermally or chemically, a monomer or polymer solution containing the enzyme. Contrary to the other immobilisation methods, this procedure can be applied to every kind of enzyme. However, a disadvantage of this method is that a large pore size could cause enzyme leakage whereas a small pore size could prevent the diffusion of large substrate molecules into the matrix to reach the biocatalyst.

3.2.6 Microencapsulation

In microencapsulation semipermeable polymer membranes are used to enclose the enzyme in microcapsules. The usual membrane pore size ranges from 1 to 100 nm, which is sufficient to prevent enzyme or cell leakage and to allow substrates to dialyse freely across the membrane. Microcapsules can be likened to artificial cells. The use of permeated, whole dead cells is a closely related technique. Another method for enzyme encapsulation is their confinement in liposomes (a liposome is an artificially-prepared vesicle composed of a lipid bilayer) (Cioci and Lavecchia, 1995)

3.3 Choice of immobilization method

The selection of an immobilisation method and of support depends on the nature of the biocatalyst (e.g., whole cells or purified enzymes), the process conditions, the type of reactor to be used, and the specific application of the biocatalyst (van de Velde, 2002). In general, the important characteristics of the immobilized biocatalyst to use are: operational stability, particle size, solubility (or insolubility), biodegradability, diffusivity of substrates and/or reactants (effectiveness factor), and in the case of cell immobilisation, the growth parameters inside the support.

Most processes in the pharmaceutical industry require small-scale operations with high research and development costs. These costs are usually recouped by charging relatively high prices for the produced drugs. In contrast, the food industry is mainly composed of large-scale operations with a low profit margin (Takata, 1980). Consumers expect a safe product, readily available for a reasonable price.

For food and pharmaceutical applications the required protocols are different than those used for chemical applications. Regulatory entities, such as the FDA, require more extensive studies when new supports, cross-linking agents, and other chemicals are introduced for enzyme immobilisation. Hence, in most cases a time-consuming effort, such as the determination of the leaching levels of materials and their toxicity, has to be carried out before the process will be FDA-approved. To save in time and costs it could be helpful to use food-grade chemicals, as well as cheap (bio)polymers generally recognised as safe (GRAS), as suitable carriers. The biodegradability of biopolymers is a major issue in cases where large amounts of biocatalyst have to be discarded.

3.4 Immobilization of enzymes on epoxy supports

Epoxy supports are one of the most suitable methods for the industrial immobilization of enzymes through covalent attachment (Hernaiz and Crout, 2000; Katchalski-Katzi and Kraemer, 2000). There are several reasons for this. They are very stable, allowing a long storage and a prolonged transport from manufacturers to consumers. Epoxy supports are reactive with different groups of proteins (amine, thiol, hydroxyl groups) yielding very stable protein-support bonds (secondary amine, thioether, ether). Furthermore, the remaining epoxy groups can be easily blocked after enzyme immobilization with different chemicals yielding an inert surface. More recently, it has been proven that epoxy supports may lead to enzyme stabilization via an intense enzyme-support multipoint covalent attachment by controlling the incubation conditions (Mateo *et al.*, 2000a; Mateo *et al.*, 2002).

3.4.1 Eupergit®: an epoxy support of industrial application

Eupergit® (Boller *et al.*, 2002) consists of porous beads carrying oxirane groups made by copolymerization of N,N′-methylene-bis-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether and methacrylamide (Figure 2).

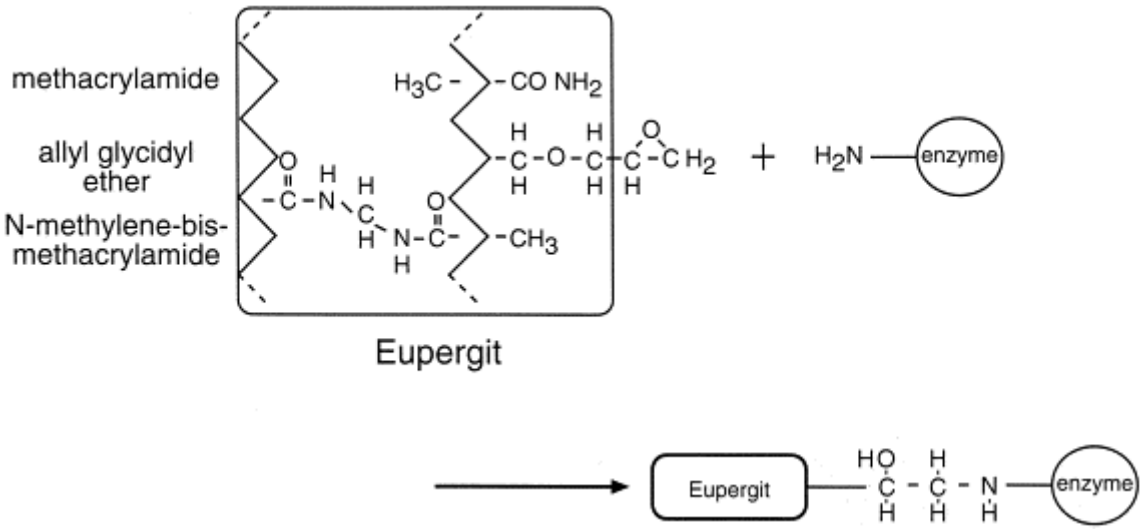

Figure 2. Chemical structure of Eupergit® showing the epoxy group of the support and the amino group of the enzyme (from Katchalske-Katzi and Kraemer, 2000).

Eupergit® is stable, both chemically and mechanically, over a pH range from 0 to 14, and does not swell or shrink. Thus, Eupergit® can covalently bind enzyme molecules practically at the pH range in which they are stable.

Eupergit® C and Eupergit® C 250 L are two commercially available forms of Eupergit®. Both forms have a diameter of 100−250 μm (Figure 3) but differ in the content of oxirane groups and in their porosity. Eupergit® C has an average pore size of 10 nm and an oxirane density of about 600 μmol/g dry beads while Eupergit® C 250 L has larger pores (100 nm) and a lower oxirane density (about 300 μmol/g dry beads). Table 3 reports the main characteristics of these two supports extracted from the literature or determined in this work. Thanks to the high density of oxirane groups on the surface of Eupergit®, enzymes are immobilized at various sites of their structure, yielding to the so-called "multi-point-attachment", responsible for the high operational stability of enzymes immobilized on Eupergit®. Enzymes can be immobilized on Eupergit® thorough a simple procedure: the enzyme is dissolved in buffer, mixed with Eupergit® and allowed to stand at 20–25°C for 24 to 100 h. The immobilized enzyme is then washed with water and buffer to be used in its subsequent application. A second incubation step with an aqueous solution of glycine or mercaptoethanol may be necessary to block the unreacted oxirane groups.

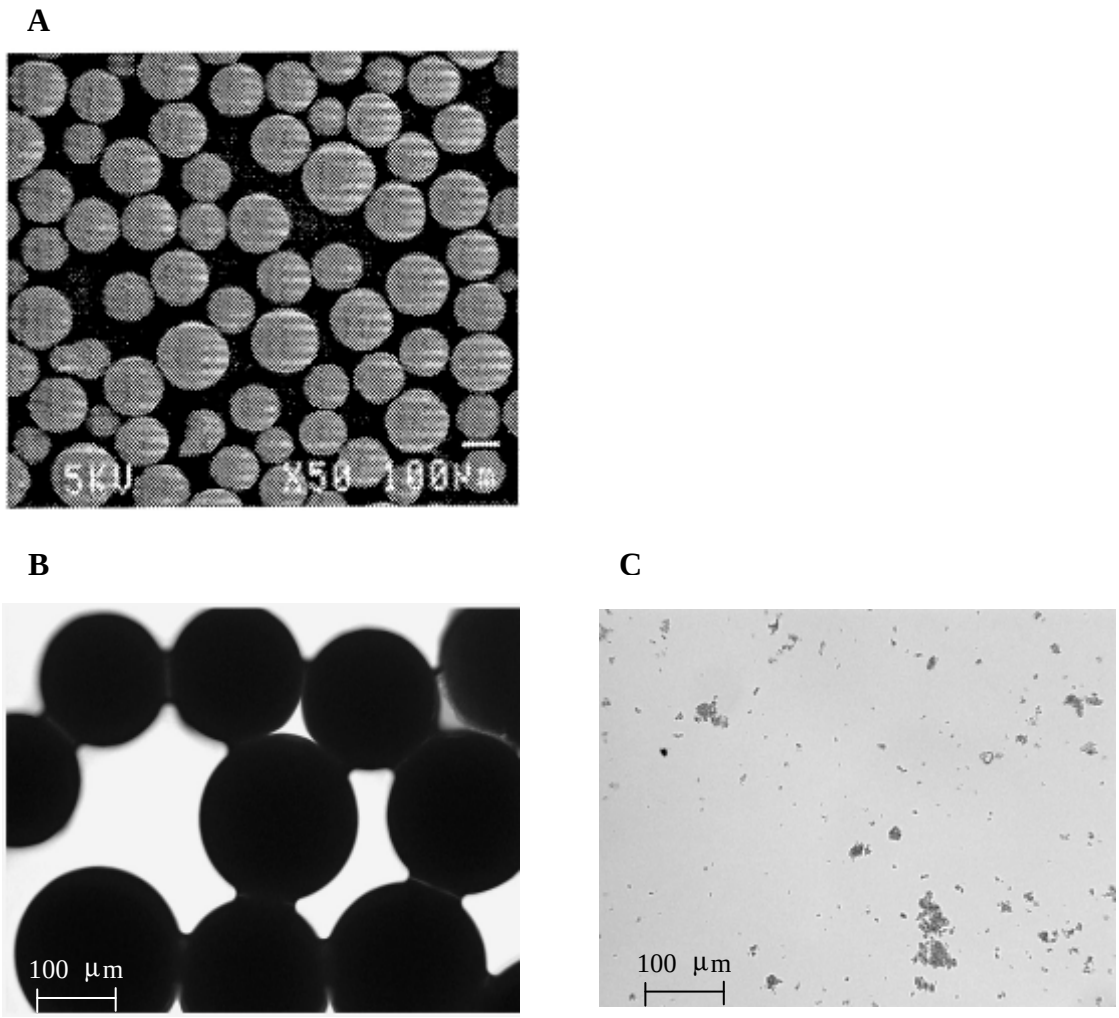

Figure 3. Images of Eupergit® beads as such obtained from Katchalski-Katzi and Kraemer (2000) (A) or from Bortone *et al.* (2012) (B) or after a crushing treatment (C) (Bortone *et al.*, 2012).

Table 3. Main characteristics of the Eupergit® C and Eupergit® C250L supports.

Parameter	Eupergit® C	Eupergit® C250L	Unit	Ref.
Average bead diameter	150	180	µm	Gómez de Segura et. al. (2004)
Average bead pore diameter	10	180-300	Nm	Spieß *et al.* (1999)
BET surface area	4.5	49.3	m^2/g ds	Gómez de Segura et. al. (2004)
Specific surface area	40,000	33,333	m^2/m^3	Bortone *et al.* (2012)
Total pore volume	0.06	1.89	mL/g ds	Spieß *et al.* (1999)
Density of epoxide groups	732±12	283±0	µmol/m^2	Bortone *et al.* (2012)
Water content	2.5±0.2	3.6±0.2	% w/w	Bortone *et al.* (2012)
Water content after swelling	70±2	75±2	% w/w	Bortone *et al.* (2012)
Bead porosity	-	0.6	-	Sundberg and Porath (1974)
Bead tortuosity factor	-	1.0	-	Sundberg and Porath (1974)
Particle density	-	0.37	kg ds/L	Spieß *et al.* (1999)

Mateo *et al.* (2000b) suggested a two-step mechanism for immobilization on epoxy supports. In the first step, the enzyme is physically adsorbed on the support by hydrophobic interactions, thus bringing amino and thiol groups on the surface of the enzyme in close proximity to the oxirane groups of the carrier. In the second step they react with the oxirane groups by nucleophilic attack. In this way, very stable C−N and C−S bonds are formed. The binding capacity of Eupergit® supports is of about 100 mg protein/g Eupergit® (dry weight).

3.4.2 Heterofunctional Chelate-Epoxy supports

The availability of pure enzymes is a key factor in the preparation of very active immobilized biocatalysts. However enzyme purification is time-consuming, requires high costs and can lead to enzyme inactivation. To overcome these problems, one possibility is to couple purification and immobilization steps by developing an immobilization process with high selectivity. Immobilized metal-chelate affinity chromatography (IMAC) is a well-developed tool at industrial scale to purify proteins fused to poly-His-tags (short tract of poly-histidine tagged to the N terminus or C terminus) (Hubert and Porath, 1980; Porath, 1992; Porath *et al.*, 1975). IMAC is mainly used for enzyme purification and not for protein immobilization because the adsorption of a poly-His-tagged protein on the chelate support is reversible and may lead to leaching of the enzyme. Also, the undesired release of metals to the reaction media may become a problem in many cases. The mechanism of enzyme immobilization in epoxy supports has led to the development of multifunctional epoxy supports to purify and immobilize proteins (Wheatley and Schmidt, 1993). These supports have different moieties that are able to physically adsorb proteins via different structural features, plus a dense layer of epoxy groups that are able to covalently react with the enzyme.

One of the types of multifunctional supports that may be easily produced are the metal chelate-epoxy supports (CES) Mateo *et al.* (2001). These supports may combine the good properties of epoxy supports for enzyme immobilization-stabilization with the high possibilities of IMAC to purify poly-His-tagged proteins (Figure 4)

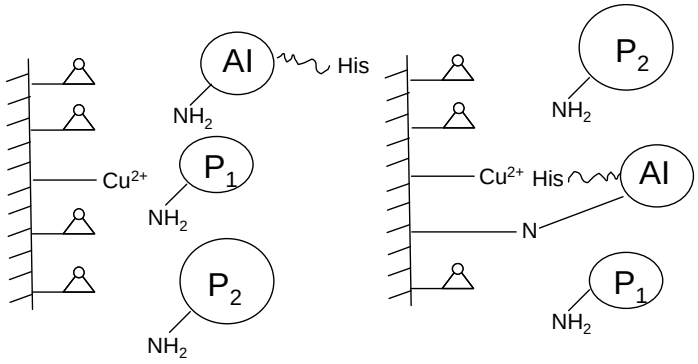

Figure 4. Proposed strategy for the purification-immobilization of a poly-His-tagged protein (AI) using copper chelate-epoxy supports. P1 and P2 represent contaminating proteins.

An example of their use may be found in the work by Pessela *et al.* (2003) and is illustrated in Figure 5 which depicts the steps involved in the development of a cobalt-chelate expoxy support. Briefly, epoxy supports are incubated in the presence of iminodiacetic acid (IDA) to introduce a few IDA groups in the support. The IDA supports are incubated with cobalt salts to obtain metal chelate-epoxy supports. These supports are used to immobilize the proteins, after which they are incubated in the presence of glycine to block the remaining epoxy groups. To eliminate cobalt from the support, an incubation with mercaptoethanol may be enough.

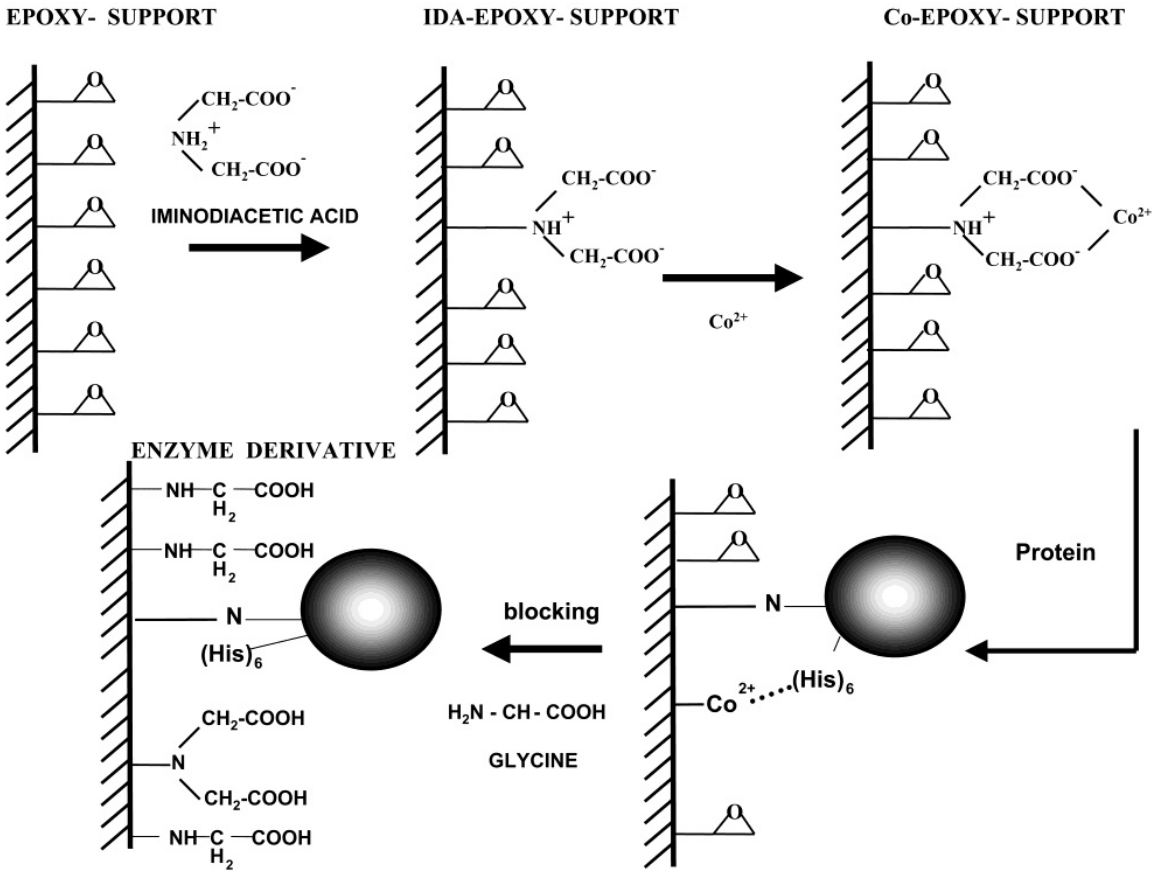

Figure 5: Steps involved in the development of a Co-chelate epoxy support for the immobilization of a poly-His-tagged protein.

3.5 Stabilization of enzymes via immobilization

Generally the improvement of enzyme stability is a desired property in immobilization. In the following section, the different levels of enzyme stabilization that can be obtained through immobilization of enzymes are briefly described with particular regard to immobilization on porous epoxy supports.

3.5.1 Operational stabilization of enzymes by immobilization on porous epoxy supports

Immobilization protocols of an enzyme which yield the enzyme immobilized inside a porous solid as the final product may lead to a stabilization of the enzyme, even without really affecting its structural stability. In fact, the immobilized enzyme molecules will not be in contact with any external hydrophobic interface, such as air bubbles originated by supplying some required gases or promoted by strong stirring. These gas bubbles may produce enzyme inactivation of soluble proteins, but cannot inactivate the enzymes immobilized on a porous

solid (Bolivar *et al.*, 2006). It is worth mentioning that this stabilization is not expected in any immobilization. For example, when using non-porous nano-particles (mainly magnetic nano-particles) to immobilize enzymes (Zeng *et al.*, 2006) all the mechanisms of enzyme stabilization described above are lost.

3.5.2 Rigidification of the enzyme structure by multipoint covalent immobilization

A rigidification of the structure of the immobilized enzyme can be obtained by the abovementioned multipoint covalent attachment of enzymes on highly activated epoxy supports via short spacer arms and involving many residues placed on the enzyme surface Mateo *et al.* (2007) (Figure 7). This rigidification does not allow to the relative distances among all residues involved in the multipoint immobilization to vary when the enzyme is exposed to distorting agents (heat, organic solvents, extreme pH values) and this reduces any conformational change involved in enzyme inactivation.

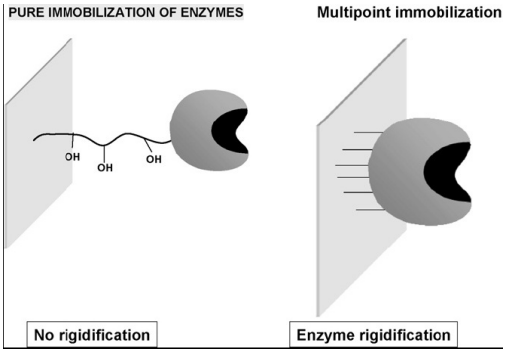

Figure 7. Effect of immobilization on enzyme stability.

These intense multipoint covalent attachments between the enzyme and the support are not easily achievable and depend on the choice of suitable immobilization systems. In same cases, the possibility of getting a significant stabilization by this technique had even been discarded, due to the only moderate geometric congruence between the enzyme and the support (Mateo *et al.*, 2007). For example, Bortone *et al.* (2012) observed that incubation of the enzyme urease immobilized on Eupergit® C250L under conditions that should favour multipoint covalent attachment between the enzyme and the unreacted epoxy groups did not result in any improvement of the stability of the enzyme. Thus, the characteristics of the support, reactive groups and immobilization conditions have to be carefully selected so that the maximum

number of enzyme groups are involved in the immobilization, this being correlated with the enzyme stabilization (Pedroche *et al.*, 2007).

The stabilization factors obtained with the immobilization of several proteins on glyoxyl agarose under optimized conditions can be extremely high (1000–10,000-fold factors) with activity recoveries usually above 60%. Lower or similar results have been obtained also with epoxy-activated supports. (see Table 4). It is important to consider that the stabilization of an enzyme by multipoint covalent attachment can be used together with any other method used to obtain a stable enzyme (protein engineering, screening, etc.). For examples, enzymes from extremophiles have been also stabilized by multipoint covalent attachment (Mateo *et al.*, 2007).

Use of glutaraldehyde is another widely used technique to immobilize enzymes by covalent attachment which has given good stabilization factors in many cases. This technique is very versatile and may be used in very different ways (Migneault, 2004; Betancor *et al.*, 2006). One possibility is the treatment with glutaraldehyde of proteins previously adsorbed on supports bearing primary amino groups. This allows the crosslink between glutaraldehyde molecules bound to the enzyme and glutaraldehyde molecules bound to the support leading to enzyme stabilization. However, it implies the chemical modification of the whole enzyme surface (Lopez-Gallego *et al.*, 2005).

Table 4. Immobilization-stabilization of several enzymes on epoxy supports. Yields based on percentage of immobilized protein that retains enzyme activity. Stabilization factor calculated with respect to the soluble enzyme (adapted from Mateo *et al.*, 2007).

Epoxy support	Enzyme	Stabilization factor	Reference
pHEMA-GMA	Cholesterol oxidase	2.5 (50ºC)	Akgol *et al.* (2002)
Poly(HEMA-GMA-2)	Alpha-amylase	2 (70ºC)	Bayramoglu *et al.* (2004)
pHEMA-GMA	Invertase	2 (70ºC)	Danisman *et al.* (2004)
Eupergit® C	Dextransucrase	30 (30ºC)	Gomez De Segura *et al.* (2004)
Eupergit® C 250L	Dextransucrase	40 (30ºC)	Gomez De Segura *et al.* (2004)
Eupergit(R) C250L	Urease	1-1.5 (65°C)	Bortone *et al.* (2012)

3.5.3 Stabilization of multimeric enzymes by multisubunit immobilization

The use of a multimeric enzyme poses some specific problems. The dissociation of these enzymes in their individual subunits (Poltorak *et al.*, 1998) can affect adversely the enzyme stability, and contaminate the final product (e.g., a food). Immobilization is a simple strategy to deal with this problem. In this case the immobilization–stabilization strategy should consider this multimeric nature of the enzyme, pursuing not only the multipoint attachment of the protein, but also the multisubunit attachment. The multimeric structure of dimeric enzymes has been easily stabilized by immobilization on highly activated supports (Fernandez-Lafuente, 1999) (Figure 8). In the case of more complex enzyme structures, it is quite likely that not all the subunits of the enzyme may be attached to a plane surface (Figure 9).

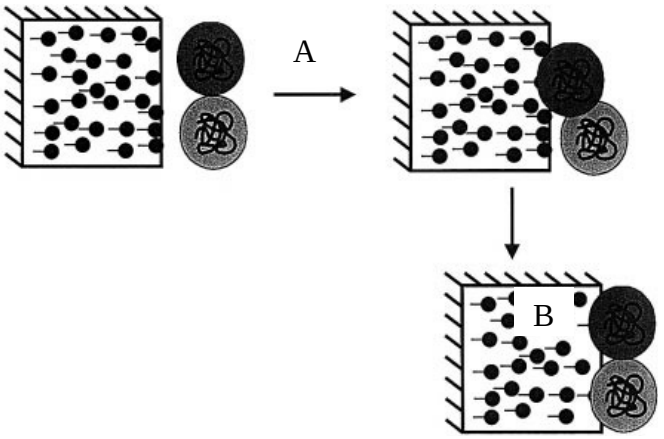

Figure 8. Stabilization of dimeric enzymes by multisubunit stabilization: the dimeric enzyme is immobilized on the support under mild conditions (A) and undergoes a multisubunit stabilization when incubated under conditions that favour multipoint covalent attachment (B).

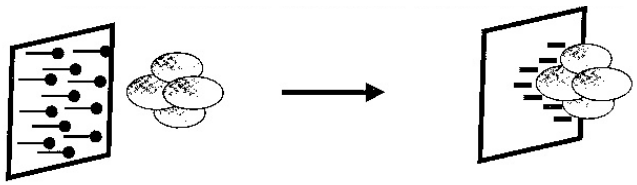

Figure 9. Multisubunit covalent immobilization.

The difficulty of stabilizing the quaternary structure of a complex multimeric enzyme immobilized on an epoxy support under conditions that should favour multipoint covalent attachment was observed also by Bortone *et al.* (2012) with the enzyme urease immobilized on Eupergit® C250L. The authors attributed the unsuitability of the immobilization system to stabilize the enzyme to geometrical incongruence between urease and Eupergit C250L. The authors argued that the pore diameter (180-300 nm) of Eupergit ® C 250 L (Table 3) is much larger than the diameter of urease (~ 8 nm). In the circumstances, each molecule of urease should see the support internal surface as a plane, this making geometrically impossible for all the enzyme subunit to establish a connection with the support.

The effect of the internal support morphology was also described by Fernández-Lafuente *et al.* (1999) who immobilized *E. coli* extracts on highly activated amino-supports, such as Toyopearl support formed by very thin fibres with approximately the same protein dimensions, and Sepabeads support having a large internal surface with pore size of about 20 nm. While after the heat treatment (used to release subunits not directly or indirectly linked to the support) Sepabeads derivatives released only some protein subunits, Toyopearl derivatives freed many proteins, thus signifying that the morphology of the support surface plays a role in the stabilization of the quaternary structure of proteins.

4. Materials and Methods

4.1 Materials

Eupergit® C 250 L (lot no. B060519592) was kindly provided by Röhm GmbH (Darmstadt, Germany). The water content of the beads as such or after 2 h of swelling in 0.05 M sodium phosphate buffer, pH 7.0 (SPB7) was determined by oven drying at 80 °C for 72 h. The oxirane content of both supports was assayed by using Sundberg and Porath's method (1974). More specifically, 300 mg of beads were suspended under gentle stirring in 15 ml of deionised water, the pH being adjusted to 7.0 with 0.01 M HCl. After addition of 15 cm^3 of an aqueous solution of $Na_2S_2O_3$ (1.3 M), the suspension was stirred for 2 h to allow the reaction to complete. Then, the OH^- ions were back titrated with a 0.01 M HCl solution to determine the bead oxirane content. Such procedure was replicated twice.

All reagents used in this work were of analytical grade.

4.2 Gene insert, plasmid and microorganism

The expression vector was supplied by Genscript Corp. (NJ, USA). Based on the published amino acid sequence of TMAI (Genbank accession number AAD35365.1, Benson *et al.*, 2009), a corresponding DNA sequence optimized for *E. coli* was synthesized. The OptimumGene™ algorithm, proprietary of Genscript Corp., used for gene optimization, takes into consideration a variety of factors that regulate and influence gene expression levels. In particular, the native gene employs tandem rare codons that can reduce the efficiency of translation or even disengage the translational machinery. The codon usage bias in *E. coli* was changed by upgrading the CAI (Codon Adaptation Index) from 0.59 to 0.94. GC content and

unfavourable peaks were optimized to prolong the half-life of the mRNA. The Stem-Loop structures, which impact ribosomal binding and stability of mRNA, were broken. In addition, the optimization process screened and successfully modified some negative cis-acting sites.

```
CATATG
ATCGATCTGAAACAGTATGAATTTTGGTTCCTGGTGGGTAGCCAGTATCTGTACGGCCTGGAAACCCTGAAAAAA
GTTGAACAGCAGGCCAGTAAAATTGTGGATAGCCTGAACGATGATCCGATTTTTCCGTCTAAAATCGTTCTGAAA
CCGGTGCTGAAAAGCTCTAGTGAAATCACGGAAATCTTTGAAAAAGCAAATGCCGATCCGAAATGCGCGGGTGTT
ATTGTGTGGATGCATACCTTCAGCCCGTCTAAAATGTGGATTCGCGGCCTGAGTATCAACAAAAAACCGCTGCTG
CATCTGCACACGCAGTATAATCGTGAAATTCCGTGGGATACCATCGATATGGATTACATGAACCTGAATCAGAGC
GCGCATGGCGATCGCGAACATGGCTTTATCCACGCACGTATGCGCCTGCCGCGTAAAGTGGTTGTGGGTCACTGG
GAAGAAAAAGAAGTTCGCGAAAAAATTGCGAAATGGATGCGTGTGGCCTGTGCAATCCAGGATGGTCGTATGGGC
CAGATTGTGCGCTTCGGCGATAACATGCGTGAAGTTGCGAGTACGGAAGGTGATAAAGTGGAAGCCCAGATTAAA
CTGGGCTGGAGCATCAATACCTGGGGCGTTGGTGAACTGGCCGAACGTGTGAAAGCAGTGCCGGAACGTGAAGTG
GAAGAACTGCTGAAAGAATATCGCGAAAAATACATCATGCCGGAAGATGAATATTCTCTGAAAGCGATTCGCGAA
CAGGCCAAAATTGAAATCGCACTGCGTGAATTTCTGAAAGAGAAAAACGCAGTTGGTTTTACCACGACCTTCGAA
GACCTGCATGATCTGCCGCAGCTGCCGGGCCTGGCCGTGCAGCGCCTGATGGAAGAAGGCTACGGTTTTGGCGCG
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TTTATGGAAGATTATACGTACCATCTGACGCCGGGCAATGAACTGGTGCTGGGCGCACACATGCTGGAAGTGTGC
CCGACCATTGCGAAAGAAAAACCGCGCATCGAAGTTCACCCGCTGAGCATTGGCGGTAAAGCAGATCCGGCGCGT
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GTGAATAAAGTTCTGAGTGTGCCGATTGAACGCAAAATGCCGAAACTGCCGACCGCCCGTGTTCTGTGGAAACCG
CTGCCGGATTTTAAACGTGCGACGACCGCCTGGATTCTGGCAGGCGGTAGCCATCACACGGCATTCTCTACCGCG
ATTGATGTGGAATATCTGATCGATTGGGCGGAAGCCCTGGAAATTGAATACGTTGTGATCGATGAAAACCTGGAT
CTGGAAGATTTTAAAAAAGAACTGCGCTGGAATGAACTGTATTGGGGTCTGCTGAAACGT
AAGCTT
```

Figure 1. Optimized sequence of TMAI gene.

Figure 1 reports the optimized sequence for the TMAI gene.

The plasmid pET-22b(+) (Cat. No. 69744-3, Novagen, USA) was used as expression vector. This vector carries an N-terminal *pelB* signal sequence for potential periplasmic localization, as well as optional C-terminal His-tag sequence. Unique sites are shown on the circle map (Figure 2). The sequence is numbered by the pBR322 convention, thus the T7 expression region is reversed on the vector map. Figure y shows the cloning-expression region of pET-22b(+).

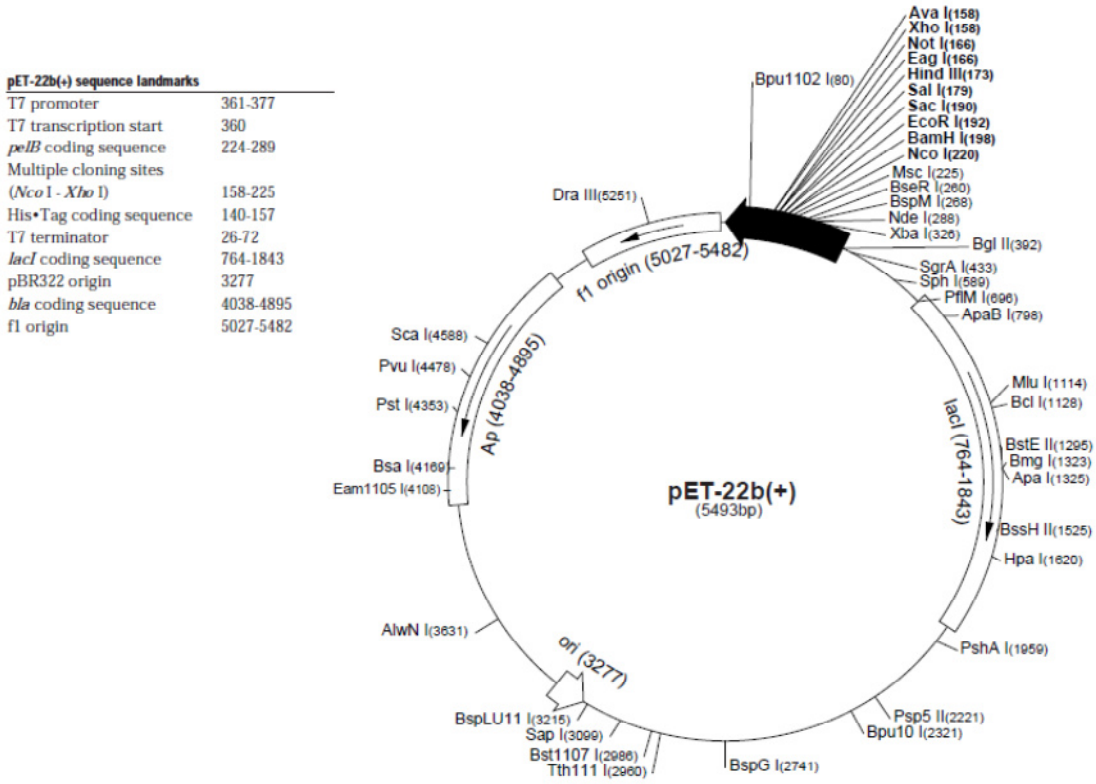

Figure 2. Circle map of pET-22b(+) vector.

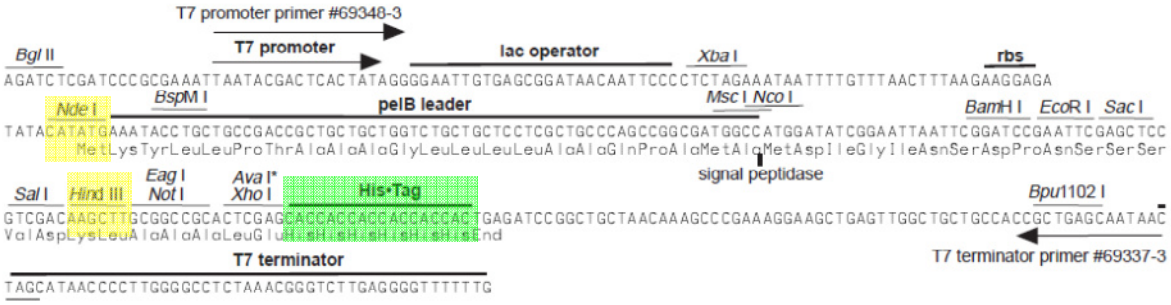

Figure 3. Cloning/expression region of pET-22b(+).

The optimized gene was ligated into the *Nde*I and *Hind*III sites of pET-22b(+) yielding the expression vector pET-TMAI encoding also for a C-terminal six-histidine sequence. The expression host *E. coli* BL21(DE3) (New England Biolabs, USA) was transformed with pET-TMAI. Induction was performed with with Isopropyl β-D-1-thiogalactopyranoside (IPTG) at final concentrations of 0.1-1 mM and at preset values of OD_{600} according to the procedures described below.

In order to check for the successful ligation of the optimized gene several an agarose gel was run with pET22b(+) and pET-TMAI undigested or digested with NdeI and HindIII.

4.3 Culture vessels, media and culture conditions

In preliminary experiments aimed at checking expression and purification of TMAI and at screening culture conditions, *E. coli* BL21(DE3) was cultivated in 250-ml baffled shake flasks on an orbital shaker (Figure 4). For the production of larger quantities of protein, *E. coli* BL21(DE3) was cultivated in 2-l jacketed stirred bioreactors (Figure 5).

Figure 4. 250-ml shake flasks filled with 50 ml of culture incubated in the orbital shaker.

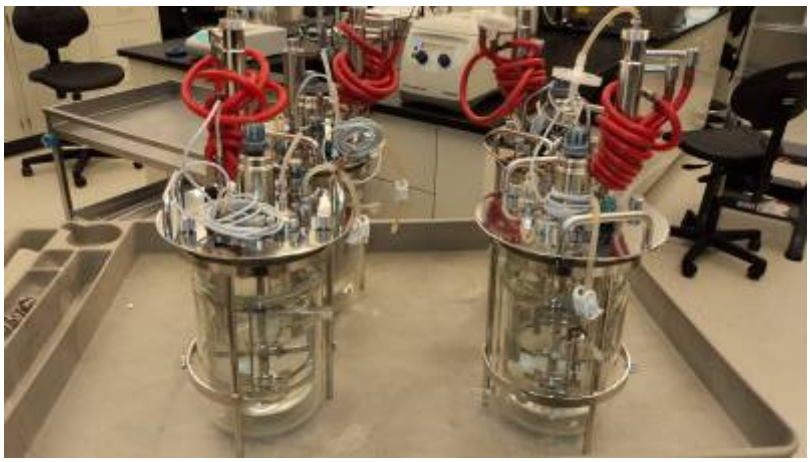

Figure 5. 2-l stirred bioreactors used for TMAI production.

Two different media were used. The first medium was Laura-Bertani (LB) medium with the following composition: Bacto tryptone, 10 g/l; Bacto yeast extract, 5 g/L; NaCl, 10 g/l. The second medium (TB) used was a rich medium with the following composition: Bacto yeast extract, 25 g/l; tryptic soy broth, 15 g/l; NH_4Cl, 1 g/l; Na_2HPO_4, 6 g/l; KH_2PO_4, 3 g/l; glucose, 10 g/l. Antifoam 204 (Sigma-Aldrich Corp, St. Louis, MO, USA) at 0.2 ml per liter of medium was added when the medium was used for the 2-l bioreactor.

The media were sterilized in glass bottles or directly in the 2-l bioreactor. For the TB medium, glucose was sterilized in a feed bottle separately from the rest of the ingredients and was transferred to the culture vessel prior to inoculation. Filter-sterilized ampicillin at a final concentration of 100 μg/ml was added to both shake flasks and bioreactor cultures prior to inoculation.

Culture conditions for shake flasks and bioreactors with LB or TB medium are reported in Table 1. In general, the culture vessel was inoculated with an overnight seed culture in LB to reach an initial OD_{600} of 0.1 and cells were allowed to grow at 37°C. When the OD_{600} reached a preset value (induction OD_{600}) the cells were induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final preset concentration and allowed to grow at 37°C or at 30 °C (induction temperature). A 1-ml sample of culture was withdrawn at the time of induction and at preset time intervals from induction to measure OD_{600} and to perform SDS-PAGE analysis. At the end of the induction phase, cells were harvested by centrifugation at 12000g for 10 min at 4°C and the cell pellet stored at -20 °C.

Table 1. Media, culture vessels, growth (dissolved oxygen, temperature, aeration flow rate, agitation speed and culture volume) and induction conditions (induction OD_{600} or induction time, IPTG concentration, temperature and duration of induction).

Medium	Culture vessel	Growth	Induction
LB Bacto tryptone 10 g/L Bacto yeast extract 5 g/L NaCl 10 g/L	250-ml baffled shake flasks	250 rpm, 37°C, 25-50 ml of culture	OD_{600}=0.5-0.6, IPTG=1 mM, 5 h at 37°C
	2-l stirred bioreactor	DO≥30%, 37°C, 1 vvm aeration, 400-1600 rpm, 1.5 l of culture	OD_{600}=0.5-0.6, IPTG=1 mM, 5 h at 37°C
TB Bacto Yeast Extract 25 g/L Tryptic Soy Broth 15 g/L NH_4Cl 1 g/L Na_2HPO_4 6 g/L KH_2PO_4 3 g/L Glucose 5-15 g/L	250-ml baffled shake flasks	400 rpm, 37°C, 25 ml of culture	induced at 3 – 4 h from inoculation, IPTG=0.1-1 mM, 20 h at 30°C
	2-l stirred bioreactor	DO≥30%, 37°C, 1 vvm aeration, 400-1600 rpm, 1.5 l of culture	Induction at 4 h from inoculation, IPTG=1 mM, 18 h at 30-37°C

4.4 Preparation of partially purified TMAI extract

Cells from two 50-ml cultures or one 100-ml culture grown in LB medium in shake flasks were harvested by centrifugation at 12000g for 10 min at 4°C and the cell pellet stored at -20 °C. The cell pellet was re-suspended in 8 ml of 50-mM sodium phosphate buffer (pH=8) and sonicated on ice to disrupt cells by using an ultrasonic liquid processor (Model S-4000, Misonix Ultrasonic Liquid Processors, NY, USA). After cell debris was removed by centrifugation at 12000g (10 min, 4 °C), the supernatant was subjected to heat treatment at 80 °C for 20 min and centrifuged at 12000g (10 min, 4 °C) to remove heat-denatured proteins, thus obtaining a supernatant rich in TMAI. This partially purified enzyme solution, referred to as TMAI extract, was used for preparation of the immobilized enzyme or freeze-dried for later use. When cells were grown in LB or TB medium in the stirred bioreactor, because of the larger amount of broth to be treated, a bigger centrifuge was used to recover cells and to remove heat-denatured proteins and disruption of cells was obtained by using a refrigerated high pressure homogenizer (GEA Niro Soavi S.p.A., Parma, Italy). Briefly, the cell pellet from 1.5 l of LB or TB culture was re-suspended in 120-200 or 250 ml of phosphate buffer,

respectively and fed to the homogenizer. The liquid was allowed to pass two times through the homogenizer and then was discharged.

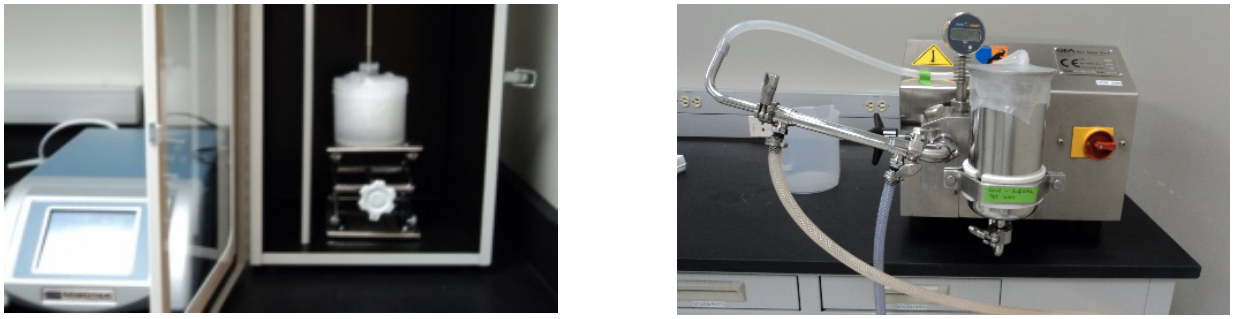

Figura 6. The ultrasonic liquid processor (Model S-4000, Misonix Ultrasonic Liquid Processors, NY, USA) (left) and the refrigerated high pressure homogenizer (GEA Niro Soavi S.p.A., Parma, Italy) (right) used for cell lyses.

4.5 Purification of TMAI by metal ion affinity chromatography (IMAC)

Purification of 6xHis-tagged TMAI was carried out by IMAC by using the QIAexpress® Ni-NTA Fast Start Kit (Qiagen, Italy). Briefly, the cell pellet from two 50-ml LB cultures was treated as described above by using 8 ml of His-binding buffer (500 mM NaCl, 20 mM Tris, 10 mM imidazole; 1 mg/ml lysozyme, pH=8) instead of the phosphate buffer. The partially purified enzyme solution thus obtained was loaded on the His-bind resin column (2 ml of wet resin) pre-equilibrated with the His-binding buffer. The column was washed with the washing buffer, and the elution buffer containing 250 mM imidazole was applied to elute the recombinant protein. The fractions containing the enzyme as monitored by absorbance at 280 nm measured by the Nanodrop^{TM} 1000 spectrophotometer (Thermo Fisher Scientific, DE, USA) were pooled and dialyzed against 20- or 50-mM Tris-HCl buffer (pH 7.9) by using the Thermo Scientific Slide-A-Lyzer G2 Dialysis Cassette with molecular weight cut-off of 7 kDa (Pierce Biotechnology, IL, USA). The dialyzed enzyme preparation was stored at 4°C and used for free enzyme activity measurements.

A B C

Figura 7. Luer lock, non-jacketed, liquid chromatography column loaded with the Ni support for IMAC (A), Thermo Scientific Slide-A-Lyzer G2 Dialysis Cassette (B) and Zeba Spin desalting columns for buffer exchange (C).

4.6 Analysis of protein fractions

Enzyme fractions for each purification step were analyzed by Experion automated electrophoresis system (BIO-RAD, USA) or submitted to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by using mini-precast 12% polyacrylamide gels and the Mini-PROTEAN Tetra Cell electrophoresis system (Bio-Rad Laboratories), following the supplier's instructions. Protein marker was supplied by Biolabs Inc. (Ipswich, MA, USA). The gels were stained with Bio-Safe Coomassie stain from Bio-rad Laboratories.

4.7 Enzyme concentration determination

TMAI concentration for all enzyme solutions was determined indirectly from D-tagatose formation rate measurements, as described in paragraph 4.12.1. The concentration of a sample of the dialyzed TMAI was estimated according to the Bradford protein assay by using the Plus protein assay kit (Pierce, USA) and the associated protein standard solution (containing 2 kg m^{-3} of bovine serum albumin, BSA). The BSA standard solution was diluted with deionised water to vary the BSA concentration in the range of 0.2-1 kg of BSA m^{-3}. By using the least squares polynomial equation of protein concentration vs. absorbance obtained for BSA, it was possible to estimate the sample TMAI concentration in terms of BSA equivalent (BSAE).

4.8 D-Tagatose determination

The amount of D-tagatose in the reaction mixture was determined by using the carbazole-cysteine method described by Dische and Borenfreund (1951) with slight modification. L-Cysteine·HCl (30 μl, 0.15 g in 10 ml of water) was added to 150 μl of sample, 0.9 ml of 9-M H_2SO_4 aqueous solution and 30 μl of 0.0120 g in 10 ml of ethanol were then added. After 10 min at room temperature the absorbance of the solution was measured at 560 nm. The concentration of D-tagatose was determined using external calibration standards with five different concentrations in the range 5-50 g m^{-3}.

The abovementioned procedure gave results affected by variability from day to day and from test to test. The accuracy of this method was subsequently improved by controlling the temperature of the reagent solutions used at 30°C and incubating the samples at the same temperature for 15 min before absorbance reading. Furthermore, the effect of different buffer systems and pH and the presence of galactose on the assay results (see section 4.13.2) was assessed systematically.

D-tagatose was also confirmed by high-performance anion-exchange chromatography with pulsed amperometric detection by using a Dionex system equipped with the CarboPac PA1 column and the ED50 electrochemical detector (Dionex Corporation, Sunnyvale, CA) by using aqueous 20-mM NaOH at 1 ml/min flow rate as eluent. Lactose was used as internal standard. During the operation of the HPLC system injections of groups of 5 samples were alternatively followed by injections of deionized water and standards to check for calibration.

4.9 Support for TMAI immobilization

The epoxy support Eupergit C250L was used as such (Eu) or chemically modified to obtain a cupper-chelate epoxy support (Eu-Cu) or a manganese-chelate epoxy support (Eu-Mn).

Cupper-chelate epoxy supports were prepared according to Mateo *et al.* (2001). Eu (0.1 g) was incubated in 1.8 ml of buffer (0.1 M sodium borate, 2 M iminodiacetic acid disodium salt [IDA]) at 25 °C for 2 h under gentle shaking. The IDA matrix thus obtained was recovered by centrifugation, washed with distilled water and further incubated in 6 ml of 33.3 g/l $CuSO_4$ aqueous solution for 2 h. Beads were finally recovered, washed with deionized water and stored at 4 °C in deionized water for later use.

Manganese-chelate epoxy supports were prepared according to the abovementioned procedure by using a 35.15-g/l $MnSO_4$ solution instead of the $CuSO_4$ solution.

For the preparation of larger quantities of IDA matrices the same mass of support/liquid ratios were used in the two steps.

4.10 Immobilization of TMAI

Several immobilization experiments were carried out in this work and are reported in Table 2.

A first set of experiments (Exp. 1-4, Table 2) was curried out to study the immobilization capacity and selectivity of the two supports (Eu and Eu-Cu) for TMAI and the effect of TMAI extract volume in the range 4-12 ml on TMAI immobilization on Eu-Cu support. An amount of wet Eu or Eu-Cu support corresponding to 0.1 g ds was suspended in a given volume of TMAI extract and incubated at 25 °C for 48 h on an orbital shaker. After incubation the biocatalyst was collected by centrifugation and washed in sequence with 5 ml of deionized water, 5 ml of 200-mM imidazole aquous solution (pH adjusted to 8 with HCl) and 24 ml of deionized water. The biocatalyst, recovered by centrifugation, was soaked in 3 M glycine at pH 8.5 at 25 °C for 24 h and following washing and recovery by centrifugation was stored at 4 °C in a storage buffer (SPB7 buffer with 20% v/v ethanol).

A second set of experiments was performed to study the immobilization capacity and activity of purified TMAI immobilized on Eu (Exp. 5-7, Table 2). In this case 3 ml of a purified enzyme solution in phosphate buffer at pH 8 and different ionic strengths (0.05, 0.33 and 0.66 M) was used with 0.040 g of dry Eu. The same procedure described above was used except for the use of only deionized water for washing the immobilized derivative.

A third set of experiments (Exp. 8, 9) was carried out to compare the immobilization capacity of Eu-Cu and Eu-Mn supports. Eu-Cu or Eu-Mn support obtained from 0.5 g of dry Eu was incubated with TMAI extract at 25°C for 48 h..

A fourth set of experiments (Exp. 10) was performed to study the effect of several post-immobilization stabilization strategies. (see the following paragraph).

The last immobilization experiment (Exp. 11) was used to prepare a large quantity of immobilized biocatalyst to be stabilized under condition A (see the following paragraph).

The effect of treatment with mercaptoetanol at a final concentration of 5% v/v in phosphate buffer (0.05 M, pH=7.75) (treatment carried out after incubation in glycine 3 M) was studied with supports labeled as 10 or 11 in Table 2.

During immobilization, supernatant samples were withdrawn for TMAI activity, total protein assay and electrophoresis analysis. Before assessing TMAI activity, supernatant samples were subjected to buffer exchange (from 50-mM sodium phosphate buffer [pH=8] to 50-mM Tris-HCl with $MnCl_2$ buffer [pH=7.5]) by using Zeba Spin desalting columns with 7 kDa molecular weight cut-off (Thermo Fisher Scientific Inc., IL, USA).

Table 2. Immobilization experiments carried out in this work. In each experiment a preset mass (M) of the support (Eu, Eu-Cu or Eu-Mn) was incubated in a volume (V_0) of immobilizing solution at a given concentration of total proteins (TP_0) and TAMI.

Exp.	Support	Source of TMAI	M (g)	V_0 (ml)	TP_0 (mg/ml)	C_{P0} (mg/ml)
1	Eu	LB medium, fresh extract	0.1	8	1.31	0.31
2	Eu-Cu	LB medium, fresh extract	0.1	4	1.07	0.34
3	Eu-Cu	LB medium, fresh extract	0.1	8	1.07	0.34
4	Eu-Cu	LB medium, fresh extract	0.1	12	1.07	0.34
5	Eu-Cu	LB medium, purified enzyme	0.040	3	0.21	0.21
6	Eu-Cu	LB medium, purified enzyme	0.040	3	0.19	0.19
7	Eu-Cu	LB medium, purified enzyme	0.040	3	0.19	0.19
8	Eu-Cu	TB medium, lyophilized extract	0.5	95	1.37	0.24
9	Eu-Mn	TB medium, lyophilized extract	0.5	95	1.37	0.24
10	Eu-Cu	LB mediu, lyophilized extract	0.5	95	0.91	0.15
11	Eu-Cu	TB medium, lyophilized extract	1	190	0.98	0.23

4.11 Post-immobilization stabilization

In order to test different post-immobilization stabilization methods, 0.5 g dry Eupergit was used to prepare Eu-Cu supports according to the procedure reported above (Exp. 8, Table 2). After the 48-h incubation of Eu-Cu supports with TMAI extract, the resulting immobilized derivatives were washed with deionized water and subjected to one of four different procedures (referred to as A-D) for their stabilization, before the final incubation in 3 M glycine. Derivatives labeled as E were treated directly with 3-M glycine for 24 h after the 48-h incubation with TMAI extract and used as a control. Below we report the five treatments tested.

A. Ethylenediamine (EDA)-glutaraldehyde. Immobilized Eu-Cu derivatives were incubated in an aqueous solution of Ethylenediamine (5% v/v; pH=10, adjusted with concentrated HCl) at 25°C for 30 min. After washing with deionized water, the derivatives were incubated in a

solution of gluteraldehyde (0.2% w/v; phosphate buffer, 0.05 M, pH 7) at 25°C for 30 min. The derivatives were then washed and treated with glycine 3 M as described above.

B. Sodium carbonate. Immobilized Eu-Cu derivates were incubated in an aqueous solution of sodium carbonate (0.1 M, pH=10, adjusted with concentrated HCl) at 25°C for 72 h. After that, the derivatives were washed with deionized water and subjected to the glycine treatment as described above.

C. Heat treatment. Immobilized derivatives were incubated in phosphate buffer (0.1 M, pH=8) at 50°C for 3 h before washing and treatment with glycine.

D. Glutaraldehyde. Immobilized derivatives were incubated in a solution of gluteraldehyde (0.2% w/v; phosphate buffer, 0.05 M, pH 7) at 25°C for 30 min. The derivatives were then washed and treated with glycine 3 M as described above.

E. Non stabilized. Immobilized derivatives were incubated directly in 3 M glycine as for the unstabilized Eu-Cu supports. This treatment was a control treatment.

Immobilized enzyme preparations corresponding to 10 mg of wet weight were suspended in 1 mL of buffer, obtained by mixing equal volumes of 0.05 sodium phosphate buffer (pH 7.0) and Laemmli sample buffer (Bio-Rad Laboratories, CA, USA) pre-added with 2-mercaptoethanol at a concentration of 5% (v/v), and heated at 95 °C for 10 min. The heat treatment in presence of sodium dodecyl sulphate (SDS) (contained in the Laemmli sample buffer) ensured the release of any protein molecule or subunit that was not, directly or indirectly, covalently linked to the support (Fernández-Lafuente *et al.*, 1999). Supernatants were submitted to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as describe above.

4.12 Determination of bound enzyme

The amount of bound protein (m_{Pb}) was indirectly assessed by subtracting the amount of protein ($c_{Pf}\, V_0$) present in the immobilising solution at the end of immobilization from the initial amount ($c_{P0}\, V_0$):

$$m_{Pb} = c_{P0}\, V_0 - c_{Pf}\, V_0 \qquad (1)$$

where V_0 is the volume of the immobilising solution and c_{P0} and c_{Pf} its initial and final TMAI concentration, respectively.

The protein loading ($Y_{P/B}$) was defined as the amount of bound protein (m_{Pb}) per unit mass of dry support (m_{Bd}) and calculated as follows:

$$Y_{P/B} = m_{Pb}/m_{Bd} \qquad \text{g bound protein/g dry support} \qquad (2)$$

where m_{Bd} is the mass of dry carrier supplied.

4.13 TMAI activity assay

4.13.1 Free enzyme

For the free enzyme, activity was assayed using 2-cm^3 Eppendorf tubes immersed in a water bath to maintain the reaction temperature at 80°C by means of a thermostat. Reaction mixtures contained 50-mM Tris-HCl (pH=7.5 at room temperature), 5 mM $MnCl_2$, D-galactose and TMAI at preset concentrations.

When using D-galactose at 4.5 g/l the above reaction mixture was referred to as standard reaction mixture (STR). The reaction was started by incubating the tubes in the water bath at 80°C and after 15-20 min of incubation it was stopped by cooling on ice. D-tagatose was determined by subjecting the reacted mixture adequately diluted with the reaction buffer to the carbazole-cysteine method.

A first set of experiments was performed with 550-650 μl of reaction mixture at D-galactose concentration of 4.5 kg m^{-3} under different concentrations of the enzyme in the range of 19.4-58.1 g BSAE m^{-3} so as to check for a linear relationship between TMAI concentration (C_{PF}) and volumetric D-tagatose formation rate (r_{TE}) to be used for indirect determination of enzyme concentration in immobilization experiments,.

A second set of experiments was carried out with 150 μl of reaction mixture to estimate the kinetic parameters of the free enzyme. In this case the enzyme concentration was kept at 120-230 g BSAE m^{-3} and D-galactose was varied in the range 1.5-300 kg m^{-3}.

In all cases, the specific enzyme activity of any solution was estimated by dividing the D-tagatose formation rate (r_{TE}) by its corresponding protein concentration (c_{PF}):

$$\pi_{TE} = r_{TE}/c_{PF} \qquad IU/g^{-1} \text{ BSAE} \qquad (3)$$

One unit of TMAI activity was defined as the amount of enzyme that produced 1 μmol of D-tagatose per min under the assay conditions.

4.13.2 Immobilized enzyme

For the immobilized biocatalysts, the enzyme activity was determined in 2-cm^3 Eppendorf tubes immersed in the water bath under constant temperature. Each tube was pre-charged with 10-60 mg of wet bead. Then 400 μl of SRM was added and the tube incubated in the water bath at the desired temperature for 10-20 min. The reaction was stopped by cooling on ice and an aliquot of the supernatant of the reaction mixture was used for D-tagatose determination.

The effect of pH in the range 4-7.5 and buffer components on immobilized TMAI activity was studied with Eu-Cu immobilized derivatives obtained under condition 3 of Table 2 by using three different buffer systems at 50 mM ionic strength: sodium acetate (pH 4 and 5.14), sodium phosphate (pH 6.25 and 7.31) and Tris-HCl (pH 6 and 7.5). Buffer pH adjustment was carried out at room temperature. Each reaction mixture contained 4.5 kg m^{-3} of D-galactose and 5 mM $MnCl_2$.

The effect of temperature at the three levels of 60, 70 and 80°C on the activity of immobilized TMAI was determined with the immobilized derivatives obtained under condition 2 (Table 1) with the SRM.

The specific activity of any immobilized biocatalyst was estimated by dividing the D-tagatose formation rate (r_T) by the concentration of dry biocatalyst (c_{Bd}) or bound enzyme ($c_{Pb}=Y_{P/B}\ c_{Bd}$) as:

$$\pi_{TB} = r_T/c_{Bd} \qquad \text{IU } g^{-1} \text{ dry support} \qquad (4)$$

$$\pi_{TBE} = \pi_{TB}/Y_{P/B} \qquad \text{IU } g^{-1} \text{ bound enzyme (BSAE)} \qquad (5)$$

Eu-Cu immobilized derivatives tested at different pH and buffer components as described above were incubated also for long times (1320 min) in order to test their operational stability.

The activity of Eu-Cu immobilized derivatives subjected to post-immobilization treatments (A-E) was studied in activity experiments with incubation times of 20 min or 860 min. Furthermore, the activity was tested in tris-HCl buffer at pH 7.5 in presence of $MnCl_2$ or $MgCl_2$ or in absence of both salts.

Heat-induced non-enzymatic isomerisation was accounted for in determining activity of TMAI by comparing absorbance readings of samples from reaction mixtures incubated in presence and absence of the biocatalyst.

4.14 Thermal stability assays

The thermal stability of free and immobilized enzyme was assessed at 80 and 90°C.

For the free enzyme, a series of 2-cm^3 Eppendorf tubes were filled with 50 μl of reaction mixture containing the purified enzyme in 50-mM Tris-HCl buffer (pH=7.5) with 5-mM $MnCl_2$ and conditioned at the above temperatures using the water bath. At different times (0-3 h) the tubes were removed and quickly cooled on ice before determining the residual activity of the free enzyme at 80°C. To this aim, the tubes containing the enzyme residual activity were added with 100 μl of D-galactose in Tris-HCl buffer with $MnCl_2$ so that its final concentration was 4.5 g/l and the reaction was carried out by incubation at 80°C for 20 min as described before.

For the immobilized enzyme, given amounts (10-40 mg) of wet bead (sample 2, Table 1) were suspended in 390 μl of SRM in Eppendorf tubes, that were submerged into the water bath at the above temperatures. At different times in the range 0-5 h the tubes were removed and quickly cooled on ice before determining the residual activity of the immobilized enzyme at 80°C and 4.5 g/l D-galactose by addition of a solution of D-galactose (10 μl).

4.15 Repeated batch bioconversion experiments

In order to study the operational stability of the immobilized biocatalyst, repeated long-time bioconversion experiments with the immobilized enzyme were performed at two levels of temperature of 60 or 80°C and at initial D-galactose concentration of 4.5 and 18 g/l or 18 g/l respectively.

The experiments at 60°C were carried out with sample in capped 10-ml conical tubes containing 2 ml of reaction mixture (50 mM Tris-HCl buffer with 5 mM $MnCl_2$) with 190 or 176 mg of Eu-Cu (sample 4, Table 2) at 4.5 or 18 g/l of D-galactose, respectively. The tubes were incubated on an orbital shaker under gentle shaking. Samples of 30 μl were withdrawn during incubation for D-tagatose concentration determination. After 32.1 and 93.4 h of incubation, at the end of the first and second cycle, respectively, the immobilized biocatalyst was recovered by filtration, washed with deionised water and suspended again in 2 ml of fresh reaction mixture to repeat the reaction for a total of three cycles.

In the experiment at 80°C, a luer lock, non-jacketed, liquid chromatography column (0.7 x 10 cm) (Sigma Chemical Co., St. Louis, MO) was used to contain the immobilized enzyme. Column temperature (measured by a portable thermometer) was maintained at 80°C by immersion in a water bath. The column was loaded with 369.5 (sample 3, Table 2) or 1780 mg of Eu-Cu (sample 10, Table 2) stabilized under treatment A and treated with mercaptoethanol and 3.6 or 4 ml of 50-mM Tris-HCl buffer (pH=7.5) with 5 mM of $MnCl_2$,

respectively. The two biocatalysts exhibited an initial activity of 5.4±1.1 or 0.71±0.06 IU/g ds, corresponding to a total immobilized activity of 2.00 and 1.26 U, respectively. The column was equilibrated at 80°C. Then the reaction was started by adding 0.4 ml of a D-galactose solution in buffer so as to set the inititial D-galactose concentration at 18 g/l and the flow recirculation was started at a volumetric flow rate of 1.4 ml/min. Samples of 10 µl were withdrawn during time for D-tagatose determination. After about 6-8 h, the reaction was stopped by discharging the liquid and cold buffer was circulated through the column. The biocatalyst was finally washed with deionised water and the cycle repeated again for a total of three cycles.

5. Results and discussion

5.1 Expression vector checking

Plasmid DNA was obtained by miniprep from transformed *E. coli* cells and a restriction analysis was carried out. pET22b(+) and pET-TMAI undigested or digested with the restriction enzymes NdeI and HindIII were run on an agarose gel (Figure 1) which showed the presence of the 1.5-kb TMAI fragment inserted in the right position.

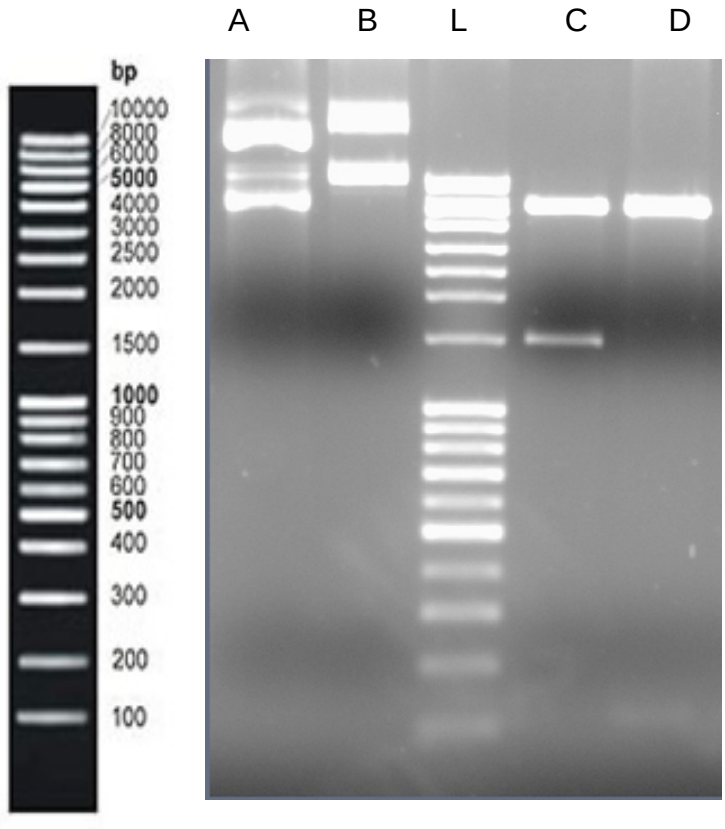

Figure 1. Agarose gel of pET22b(+) and pET-TMAI: lane A, undigested pET22b(+); lane B, undigested pET-TMAI; lane l, ladder; lane C, pET-TMAI digested with NdeI and HindIII; lane D, pET22b(+) digested with NdeI and HindIII. The fragment in the digested pET-TMAI (lane C) at about 1.5 kb corresponds to the TMAI gene.

5.2 Selection of expression colonies

Four single freshly transformed colonies were picked from the transformation plate and spread onto four fresh plates. From each plate a single colony was picked and transferred into a tube with 3-ml of LB medium with ampicillin and grown overnight at 37°C. Each culture was used to inoculate 25 ml of LB medium plus ampicillin and allowed to grow at 37°C in a 125-ml shake flask. The selected colonies were checked for protein expression levels using IPTG-induced expression. Figure 2 shows the SDS-PAGE analysis of protein expression by the four colonies while Figure 3 shows the time course of biomass growth. It can be noted that TMAI, corresponding to the band indicated by the arrow, was overexpressed in all cases. By analysing the two gels it was decided to select colony # 2 and to harvest the cells for protein purification after 5 h from induction. The selected colony was used for preparation of glycerol stocks which were stored at -80°C in a freezer.

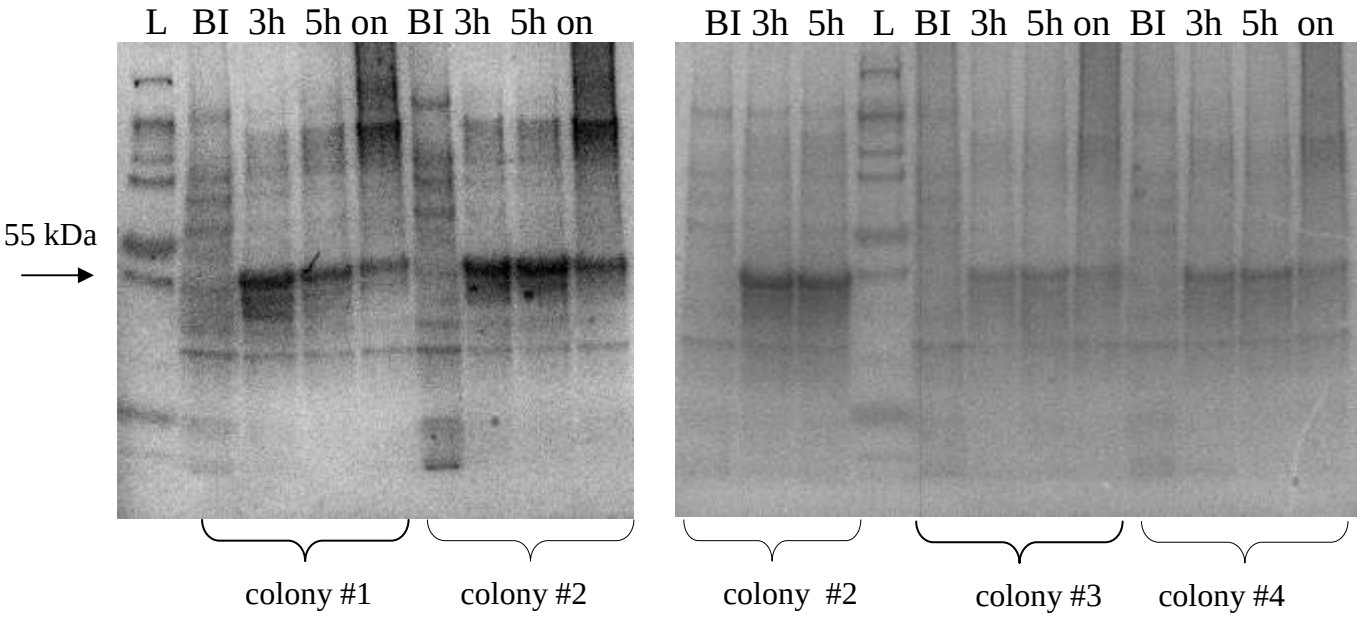

Figure 2. SDS-PAGE of protein expression in LB medium in shake flasks starting from four different colonies from the transformation plate: L, ladder; BI, cell pellet before induction; 3 h, 5 h and on, cell pellet recovered after 3 h, 5 h or after an overnight (≈18 h) from induction. The arrow indicates the 55-kDa ladder band corresponding to TMAI molecular weight. The same amount of biomass was loaded in each well.

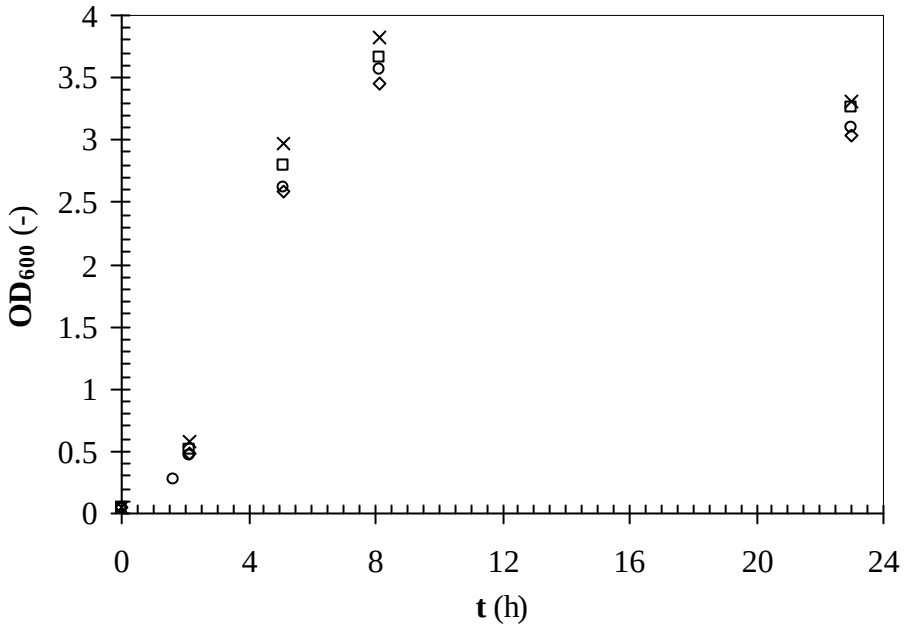

Figure 3. *E. coli* biomass expressed as optical density at 600 nm (OD_{600}) as a function of time (t) for the expression experiments carried out in 125-ml baffled flasks with 25 mL of LB medium incubated at 37°C and 250 rpm: ○, colony # 1; ◊, colony #2; x, colony #3; □, colony #4.

5.3 Expression experiments in LB medium

Several growth and expression experiments were carried out in shake flasks in LB medium according to Table 1 of the section Materials and Methods. Figure 3 reports a typical growth curve for *E. coli* cells induced at OD_{600} of about 0.6 with 1 mM IPTG used for TMAI production. The maximum OD_{600}, corresponding in many cases to the OD_{600} of harvesting, varied in the range 3-4.

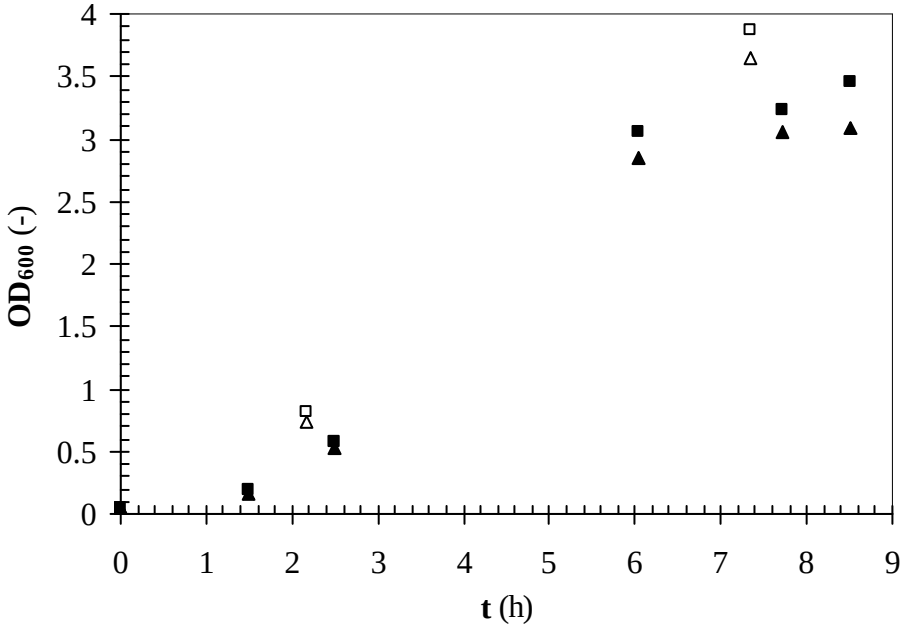

Figure 4. E. *coli* biomass expressed as optical density at 600 nm (OD_{600}) as a function of time (t) for the expression experiments: 250-ml baffled shake flasks with 50 ml of LB medium (closed symbols), 500-ml baffled shake flasks with 100 ml of LB medium (open symbols), incubated at 37°C and 250 rpm. Induction was performed with 1 mM IPTG at OD_{600}=0.6.

Figure 5 reports the time course of pH, dissolved oxygen (DO) and biomass expressed as OD_{600} measured during the batch fermentation of *E. coli* in LB medium in the 2-l stirred bioreactor. It can be observed that oxygen was not limiting, the dissolved oxygen controller being able to assure a dissolved oxygen concentration higher than 30%. It is interesting to note that from a biomass growth point of view the cultures obtained in shake flasks and in the bioreactor were equivalent, yielding a final OD_{600} in the range 3-4, corresponding to a mean concentration of biomass in all growths, estimated as wet weight, as equal to 5.1±0.9 g/l.

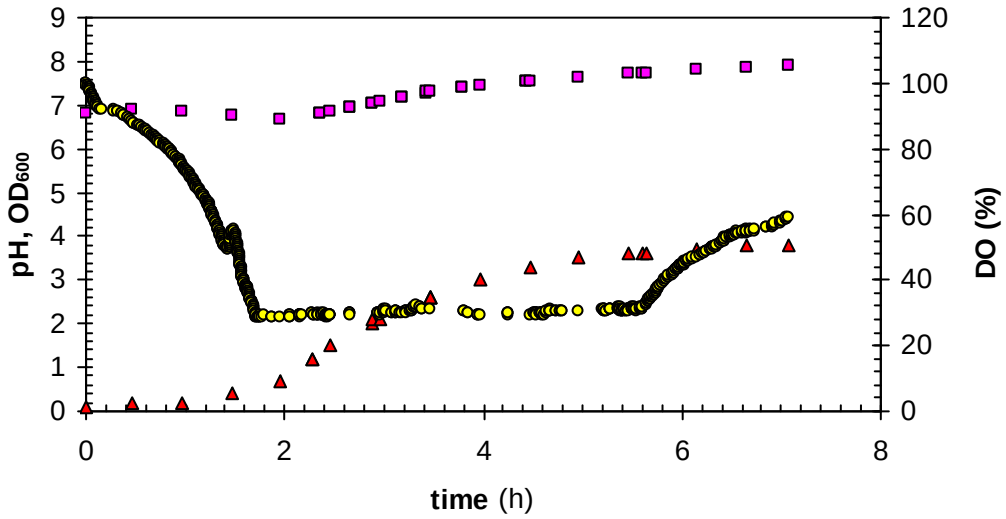

Figure 5. Time course of pH (□), dissolved oxygen (DO, ○) and biomass expressed as OD_{600} (Δ) measured during the *E. coli* batch fermentation in LB medium under the conditions listed in Table 1 of Materials and Methods.

5.4 Expression experiments in TB medium

Table 1 reports the conditions used in shake flasks expression experiments with TB medium together with overnight (about 23 h of culture time) OD_{600}.

Table 1. Shake flask expression experiments in TB medium. The experiment is a three-factor fractional factorial design with induction time (and correspondently induction OD_{600}), IPTG concentration and glucose in TB medium at two levels. Other experimental conditions are reported in Table 1 of Materials and Methods.

Experiment	Induction time (h)	Induction OD_{600}	IPTG concentration (mM)	Glucose in TB medium (g/L)	Final OD_{600}
D1	3	2.38	1	5	20.26
A2	3	2.27	0.1	15	23.7
C3	4	5.99	0.1	5	17.94
B4	4	6.25	1	15	21.72

E. coli in TB medium grew much more than in LB medium, as expected. Furthermore, it is interesting to note that the use of baffled shake flasks together with low filling volumes (10% of flask volume) and high agitation rate (400 rpm) allowed the cultures to reach very high OD_{600} values (20.9±3.4).

The results obtained in terms of biomass growth for expression experiments with the TB medium carried out in 2-l bioreactors are reported in Table 2 while the time course of OD_{600}, dissolved oxygen and pH for a batch experiment are reported in Figure 6.

Table 2. Bioreactor expression experiments in TB medium. Each batch experiment was replicated twice at two different induction temperatures (30 and 37°C). All the experimental conditions are reported in Table 1 of Materials and Methods.

Experiment	Induction OD_{600}	Induction temperature (°C)	OD_{600} at 3 h from induction	Final OD_{600}
1	5.9	37	29.68	30.92
2	6.08	37	31.32	30.04
3	6.8	30	28.2	31.16
4	7.14	30	28.2	28.12

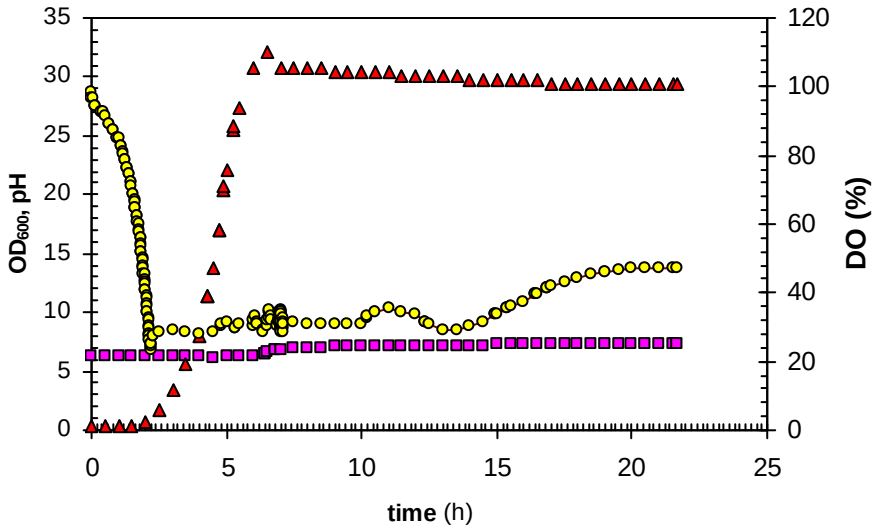

Figure 6. Time course of pH (□), dissolved oxygen (DO, ○) and biomass expressed as OD_{600} (Δ) measured during the *E. coli* batch fermentation in TB medium under the conditions listed in Table 1 of Materials and Methods. Induction temperature was 37°C.

It can be observed that, thanks to the high oxygen capability of the stirred bioreactor, the dissolved oxygen was maintained always at values ≥30%, even at the high cell densities obtained with the TB medium. On the other hand, in shake flasks oxygen might have become a limiting factor and been responsible for the lower OD_{600} obtained compared to the results in 2-l bioreactors.

5.5 Purification of TMAI

From the virtual gel of protein fractions during TMAI production and purification (Figure 7), it is possible to note that TMAI, which corresponds to the 55-kDa band, was overexpressed,

the heat treatment removed the majority of *E. coli* endogenous proteins and the purification through IMAC was successful.

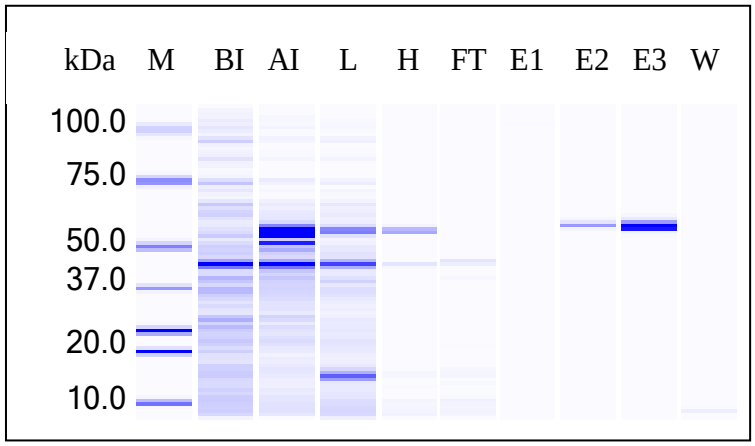

Figure 7. Virtual gel of protein fractions for the purification of TMAI by heat treatment and IMAC: molecular weight markers (M); crude extract of *E. coli* BL21 (pET-TMAI) before (BI) and after induction (AI), respectively; supernatant of lysate (L) and of heat treated fraction (H), respectively; flow trough (FT), eluates (E1-E3), and wash (W) through the IMAC column. TMAI corresponds to the~ 55-kDa band.

Several purifications were carried out to produce the TMAI used in this work. Each purification process was monitored by using either SDS-page or Experion automated electrophoresis system, absorbance measurements at 280 nm and Bradford assay. Figure 8 reports the SDS-page gel of a different purification process without the heat treatment step which confirms the results shown in Figure 7.

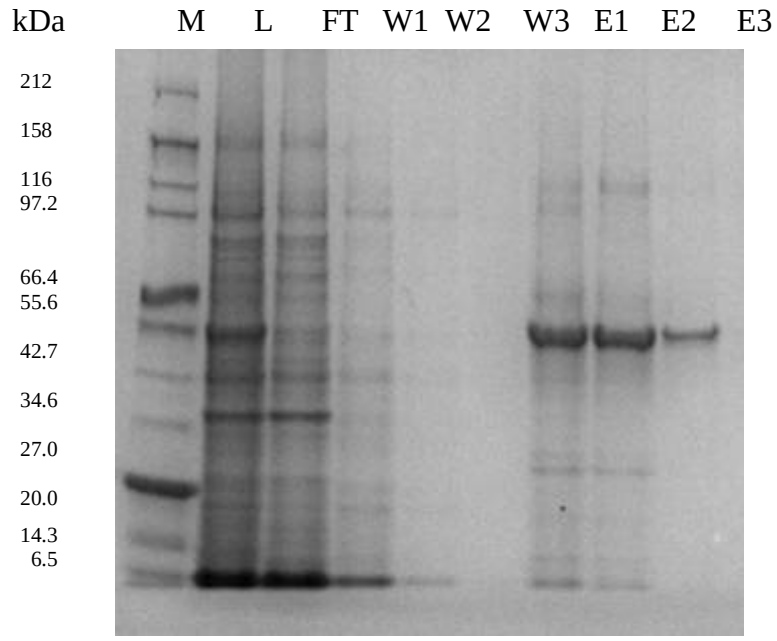

Figure 8. SDS-PAGE of protein fractions for the purification of TMAI by IMAC: molecular weight markers (M); supernatant of lysate (L) of *E. coli* BL21 (pET-TMAI); flow trough (FT), eluates (E1-E3), and wash (W1-W3) through the IMAC column. TMAI corresponds to the~ 55-kDa band.

By collecting the results of several partial purifications by heat treatment or purifications through IMAC it was possible to build Table 3 and Figure 9. From Table 3 the following mean purities estimated by the Experion automated electrophoresis system and corresponding standard deviations were estimated: 16.4±3.7 , 52.0± 8.3, 92.4± 0.8. for lysate supernatant, heat treatment supernatant and elution pool after IMAC, respectively.

Table 3. Main results of the partial purification or purification by IMAC of TMAI. V_B and OD_{600}, volume and OD_{600} of fermentation broth; V_{BA}, volume of buffer used for pellet resuspension; M_X, mass of wet biomass recovered from the broth; $P_{AI,L}$, purity of TMAI in the lysate supernatant as determined by Experion system; $M_{TP,HT}$, mass of total proteins in supernatant after heat treatment; $P_{AI,HT}$, purity of TMAI in the supernatant after heat treatment as determined by Experion system; $M_{AI,HT}$ or $M_{AI,E}$ mass of TMAI in the supernatant after heat treatment or in the elution pool after IMAC, respectively; $P_{AI,E}$ purity of TMAI in the elution pool after IMAC as determined by Experion system.

V_B	OD_{600}	V_{BA}	M_X	$P_{AI,L}$	$M_{TP,HT}$	$P_{AI,HT}$	$M_{AI,HT}$ or $M_{AI,E}^{(*)}$	$P_{AI,E}$
mL	-	mL	g	%	mg	%	mg	%
100	3.65	8	0.578	11	4.6	44	$1.2^{(*)}$	93
200	3.76	16	1.216	21	21	50	5	-
400	3.76	24	2.205	18.31	25.7	51	8.2	-
-	-	-	-	15.5	-	57.5	-	-
130	3.76	8	0.51	-	4.72	65.5	-	-
1500	3.8	120	6.607	16	106.9	44	$22^{(*)}$	91.87

From Figure 9, it can be observed that the amount of total proteins in the supernatant of heat treated samples is linearly correlated to the amount of cell paste treated. This is true also for TMAI either present in the supernatant after heat treatment or present in the elution pool.

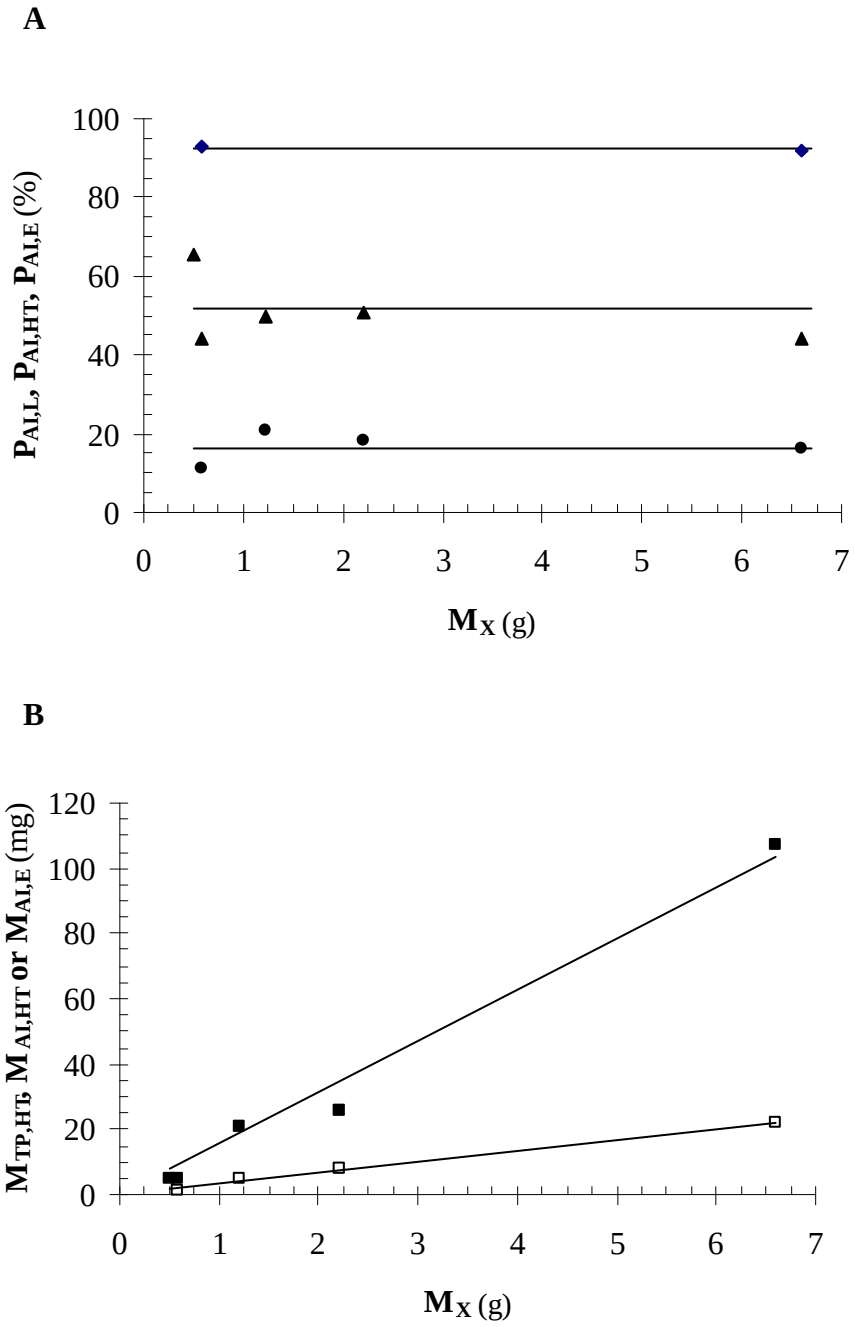

Figure 9. Main parameters characterizing the partial purification by heat treatment or purification by IMAC of TMAI: A, $P_{AI,L}$ (●), $P_{AI,HT}$, (▲), $P_{AI,E}$ (♦) purity of TMAI in the lysate supernatant, in the supernatant after heat treatment or in the elution pool after IMAC as determined by Experion system, respectively, vs. the starting mass of wet cell pellet (M_X); B, $M_{TP,HT}$, mass of total proteins in supernatant after heat treatment (■), $M_{AI,HT}$ or $M_{AI,E}$ mass of TMAI in the supernatant after heat treatment or in the elution pool after IMAC, respectively (□) vs. the starting mass of wet cell pellet (M_X).

5.6 D-tagatose determination

5.6.1 Carbazole-cysteine method

Although the carbazole-sulfuric acid method has been used extensively, we found that the results obtained by applying the original method of Dische exhibited a large variability. In order to improve its reproducibility a study was undertaken to control the different sources of variability, based on the work of Holzman *et al.* (1947). It was found that vortexing the samples after the addition of each kit reagent as well as conditioning the temperature of all reagents at 30°C and incubating the samples after the addition of the last kit reagents at the same temperature for 15 minutes reduced considerably the variability of the results. For the optimized procedure, the error between replicated assays for samples of two different concentrations of D-tagatose was equal to 1.8 mg/l of D-tagatose.

The effect of buffer components and pH was studied through a factorial experiment with two factors: concentration of D-tagatose, two levels; buffer type, six levels. Figure 10 reports the results obtained. It can be seen that the data fall on parallel straight lines suggesting that the buffer system produced only a shift of the intercepts. This was confirmed by the analysis of variance that did not reveal any main effect or interaction of the buffer on the net absorbance response ($A-A_0$, difference between the absorbance of a sample and the corresponding blank).

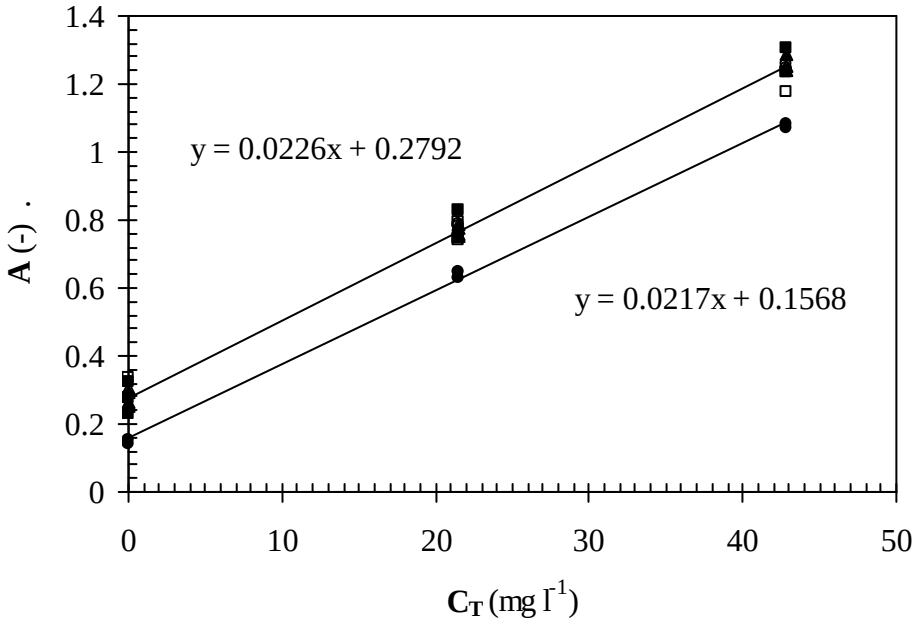

Figure 10. Absorbance readings (A) of D-tagatose solutions at several concentrations (C_T) with different buffer components and pH: phosphate buffer at pH 7.31 (●) or 6.25 (○); tris buffer at pH 7.5 (▲) and 6 (Δ); acetate buffer at pH 5.14 (■) and 4 (□). Each sample contained 4.5 g/l of D-galactose.

Further experiments were carried out to test if the presence of D-galactose had an effect on D-tagatose determination. As can be seen in Figure 11, the presence of D-galactose at 4.5 g/l produced a shift in absorbance that could be canceled out by subtracting the blank.

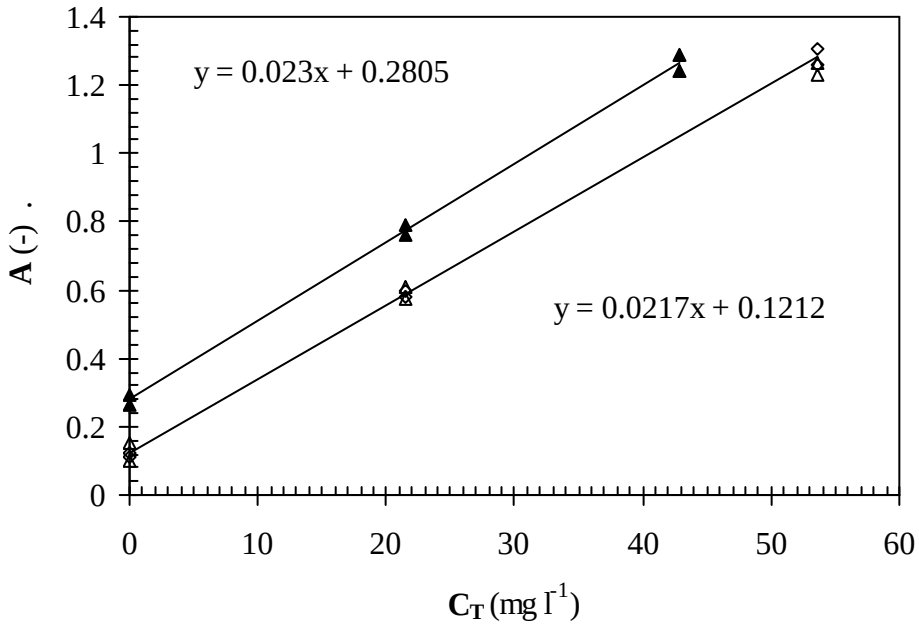

Figure 11. Absorbance readings (A) of D-tagatose solutions at several concentrations (C_T) in presence (closed symbols) or absence (open symbols) of D-galactose at 4.5 g/l with tris buffer (▲,Δ) or water (◊).

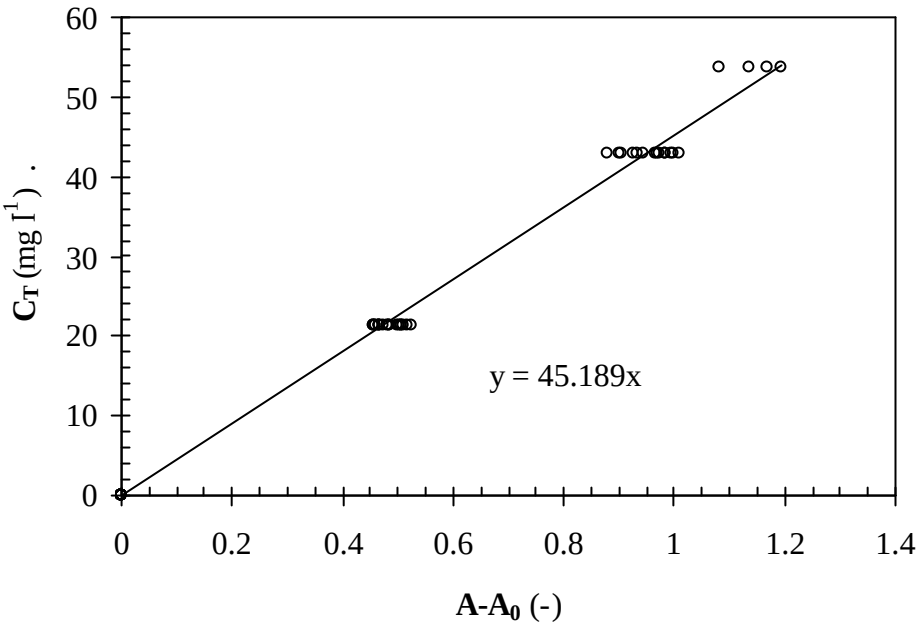

Figure 12: Concentration of D-tagatose solutions (CT) as a function of net absorbance ($A-A_0$) for all the studied systems.

By correlating all the C_T-vs-($A-A_0$) data (Figure 129 by the least-squares method the following equation was obtained:

$C_T=(45.2\pm0.3)\cdot(A-A_0)$, $r^2=0.998$. (1)

5.6.2 High performance anion-exchange liquid chromatography method

Carbohydrates can be detected by high- performance anion-exchange chromatography with pulsed amperometric detection (Cataldi *et al.*, 2000). Figure 16 reports the chromatogram of a standard solution containing D-galactose and D-tagatose. It can be seen that the shape of the peaks and the separation was quite good even though at higher concentrations the D-tagatose peak tended to be right-tailed.

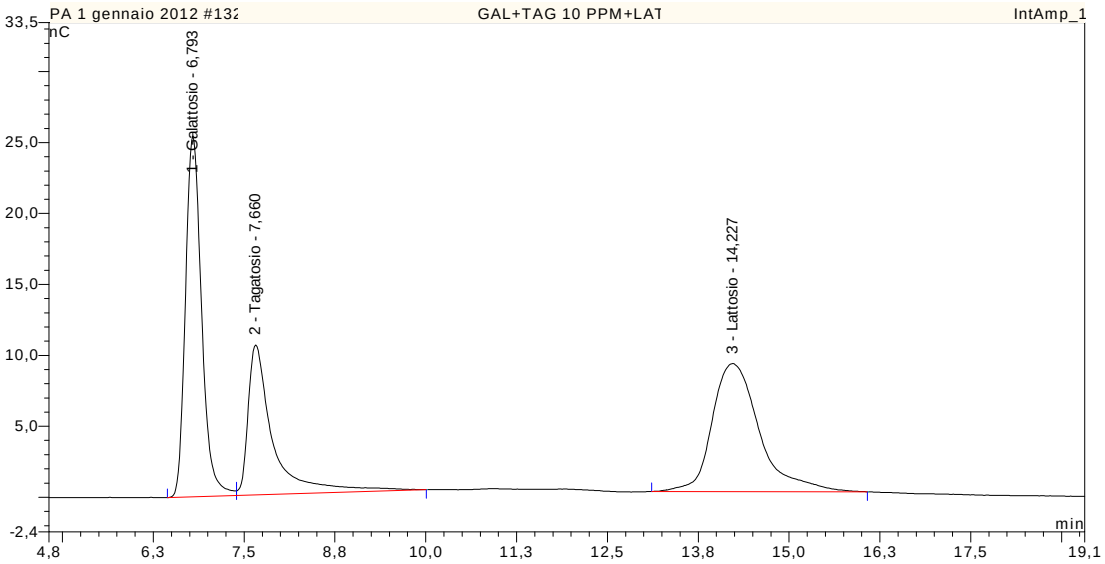

Figure 13. Chromatogram showing D-galactose, D-tagatose and lactose peaks at concentrations of 9.5, 12.6 and 20 mg/l, respectively, under the following conditions: CarboPac PA1 column with ED50 electrochemical detector (Dionex Corporation, Sunnyvale, CA), aqueous 20-mM NaOH at 1 ml/min flow rate as eluent.

By correlating the concentration data as a function of the ratio between the peak area of the sugar and that of the internal standard (lactose) for several standard solutions (Figure 14) the following regression equations were obtained:

$C_G=(12.9\pm0.4)\ A_G/A_L,\ r^2=0.997$ (2)

$C_T=(20.0\pm0.3)\ A_T/A_L,\ r^2=0.999$ (3)

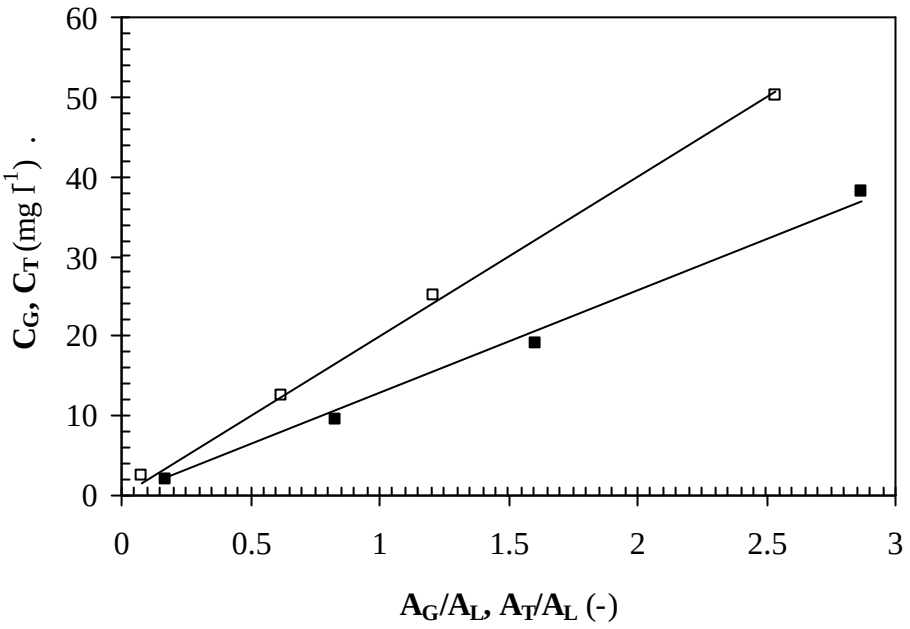

Figure 14. Concentration of D-galactose (C_G, ■) and D-tagatose (C_T, □) as a function of the ratio of their correspondent peak area (A_G or A_T) and lactose peak area (A_L).

5.7 Characterization of free TMAI

Prior to the immobilization studies, the activity of free TMAI was studied. As shown in Fig. 15, the D-tagatose specific activity (π_{TE}) exhibited a typical saturation pattern as the D-galactose concentration was increased from 1.5 to 300 g l^{-1} and was therefore fitted by using the well-known Michaelis-Menten kinetic model:

$$\pi_{TE} = \frac{k_{cat}\, S}{K_M + S} \qquad (4)$$

where k_{cat} is the maximum D-tagatose specific formation rate constant and K_M the Michaelis-Mentent constant.

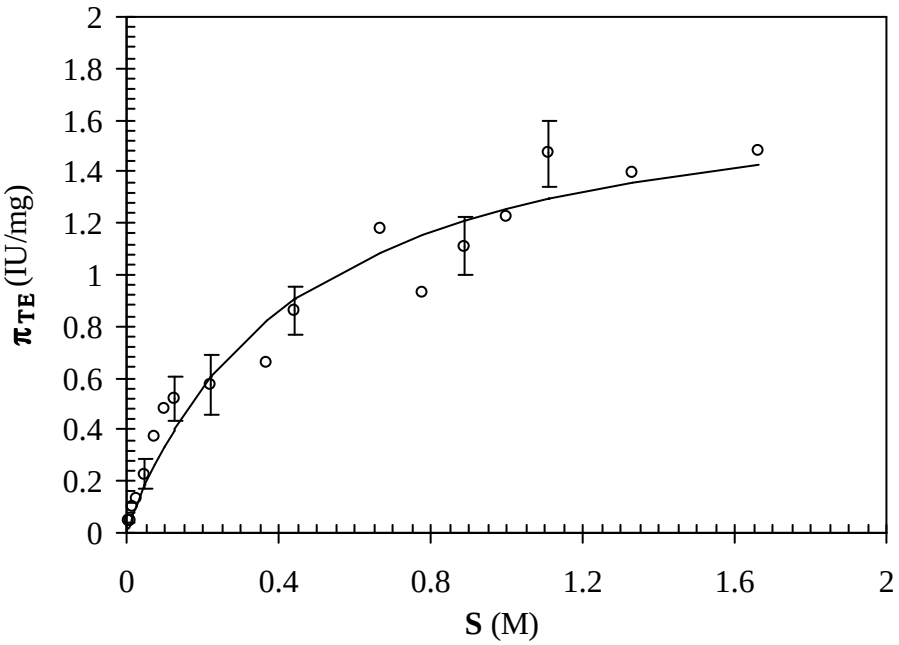

Figure 15. Effect of D-galactose concentration (S) on the specific TMAI activity (π_{TE}) at pH=7.5 (50 mM Tris-HCl, 5 mM $MnCl_2$) and 80°C. The continuous line was calculated using Eq. (4) together with the estimated parameters. Bars represent standard deviations of replicated experiments.

By referring to the specific enzyme activity (π_{TE}) and resorting to non-linear last squares method it was possible to fit the kinetic data and obtain the estimates of the mean and standard errors as k_{cat}= 1.8±0.1 IU/mg and k_M=0.43±0.09 M. TMAI was characterized by Lee *et al.* (2004) under different conditions. The authors reported k_{cat} and k_M values of 8.9 IU/mg and 0.06 M, respectively, at 90°C, pH=7-7.5 in presence of 1 mM Co^{2+} and 10 mM Mn^{2+}. This difference in the catalytic properties is most likely due to different temperature and the simultaneous presence of Mn^{2+} and Co^{2+} used by Lee *et al.*. While Co^{2+} could increase the activity of the enzyme, it was not used in this study because its presence causes potential problems in food applications.

By setting D-galactose concentration at 4.5 g/l and increasing the concentration of TMAI (c_{PF}) from about 19 to 58 g BSAE m^{-3}, r_{TE} was found to increase almost proportionally to c_{PF} (Fig. 16) thus allowing to estimate the D-tagatose formation rate (r_{TE}) at 4.5 g/l of D-tagatose as follows:

$$r_{TE} = \pi_{TE}\, c_{PF} \qquad (5)$$

where π_{TE} is the specific D-tagatose formation rate at 4.5 g/l D-galactose, estimated as the slope of the least square regression line as 0.16±0.01 IU/mg BSAE (mean ±standard error).

Eq. (5) was used to estimate indirectly TMAI concentration from D-tagatose formation rate data.

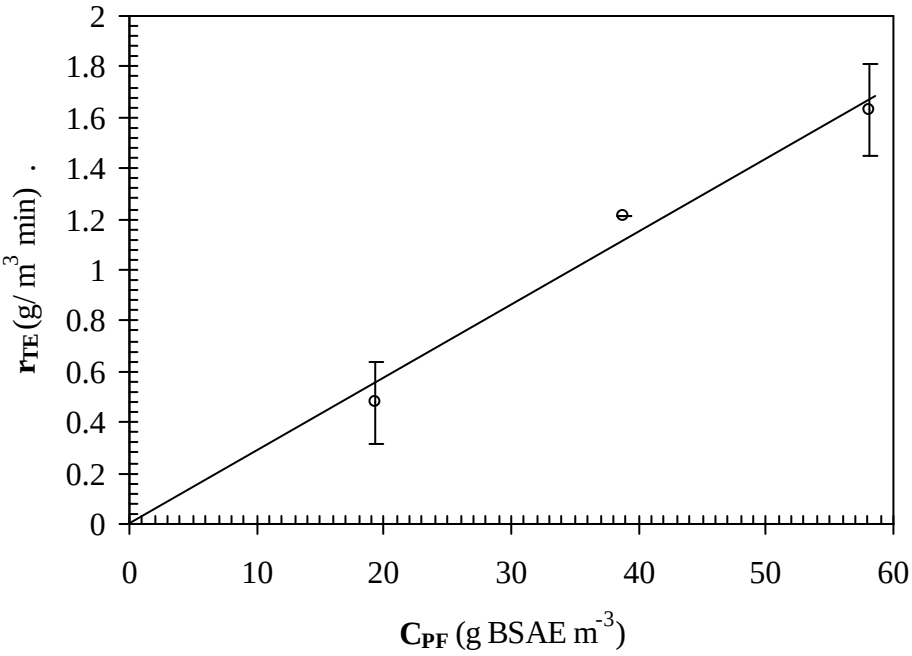

Figure 16. Effect of TMAI concentration (C_{PF}) on D-tagatose formation rate (r_{TE}) in standard reactiom mixture (50 mM Tris-HCl pH 7.5, 5 mM $MnCl_2$, 4.5 kg m^{-3} D-galactose) at 80°C. The continuous line represents the least squares regression line.

5.8 Immobilization of TMAI on Eu, Eu-Cu or Eu-Mn supports

Epoxy support modification (Figure 16) was used to create bifunctional supports, with moieties able to adsorb the His-tagged protein (Cu chelate or Mn chelate) and groups enabling protein covalent immobilization (remaining epoxy groups).

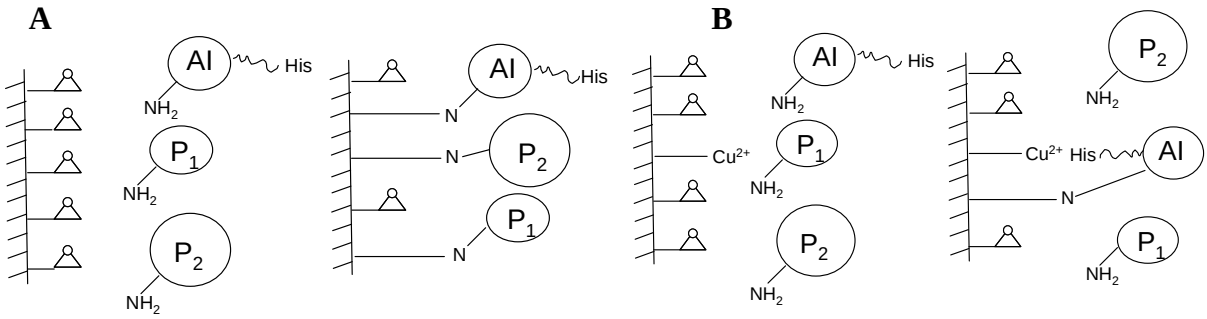

Figure 16. Schematic structure of immobilized TMAI with Eu (A) or Eu-Cu (B): AI, TMAI; P_1, P_2, endogenous *E. coli* proteins.

Table 4 reports the results of the first set of immobilization experiments carried out in this work starting from the fresh TMAI extract. The virtual gel reported in Figure 17 shows SDS protein analysis for immobilization experiments aimed at comparing the immobilization

capability of Eu and Eu-Cu (Exp. 1 and 3, Table 4). Physical adsorption on Eu appeared slow and non-selective. On the contrary, after 14 h of incubation, most of TMAI contained in the extract was physically adsorbed on Eu-Cu while other proteins remained in the supernatant, thus proving the higher selectivity of this support toward TMAI. Furthermore, after 48 h of enzyme-support interaction, most proteins remained bound to either support, not being released after washing steps, indicating covalent immobilization.

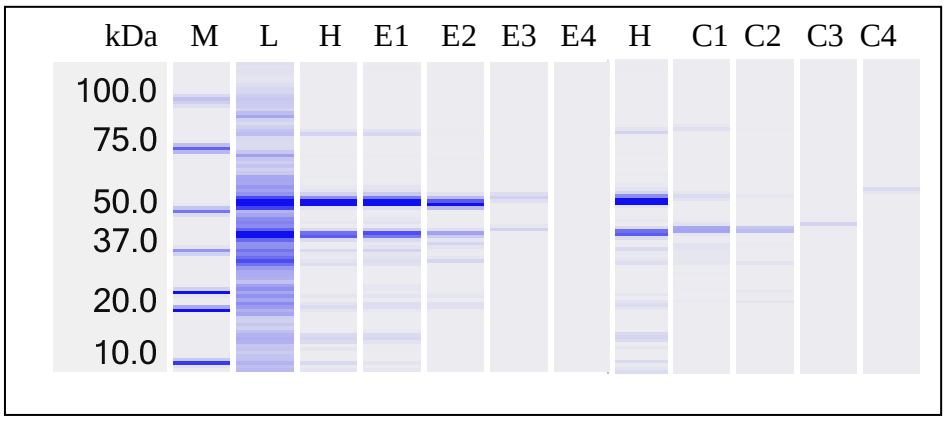

Figure 17. Virtual gel of proteins during immobilization 1 and 3 of Table 2 of Materials and Methods: molecular weight markers (M); supernatant of lysate (L) and of heat treated fraction (H), respectively; supernatants after 14 (E1, C1) or 44 h (E2, C2) of immobilization; proteins released by washing with deionised water (E3, C3) or 200 mM imidazole (E4, C4). E label refers to Eu while C to Eu-Cu supports.

Table 4. Immobilization of TMAI from extract on 0.1 g (m_{Bd}) of Eu or Eu-Cu: V_0, C_{P0}, C_{Pf}, TMAI extract volume, initial and final concentration, respectively; R, percentage of immobilization; $Y_{P/B}$, immobilization yield; π_{TB}, specific activity; Y_T, catalytic efficiency. Activity experiments were run in duplicate. Values with the same letter were not significantly different (Fisher's method applied to the natural logarithm of π_{TB} or Y_T to stabilize variances, 90% confidence level). Each data set in the table is labelled with the same number as in Table 1 of Materials and Methods. Protein concentrations were determined indirectly from activity measurements.

Exp.	Support	V_0	I	C_{P0}	C_{Pf}	R	$Y_{P/B}$	π_{TB}	Y_T
		ml	M	mg/ml	mg/ml	%	mg BSAE/g ds	IU/g ds	%
1	Eu	8	0.05	0.31	0.22	28.75	7.2	0.44 ± 0.04^{a}	39 ± 3^{a}
2	Eu-Cu	4	0.05	0.34	0.00	100.13	13.7	1.9 ± 0.4^{b}	86 ± 17^{b}
3	Eu-Cu	8	0.05	0.34	0.03	90.98	25.0	3.1 ± 0.4^{c}	77 ± 11^{b}
4	Eu-Cu	12	0.05	0.34	0.06	82.34	33.9	5.4 ± 1.1^{d}	99 ± 20^{b}

Eu-Cu incubated with the TMAI extract under the same conditions of Eu (Exp. 1 and 3, Table 4) resulted in a 3.5-fold higher immobilization yield ($Y_{P/B}$) and a 5.5-fold higher specific activity (π_{TB}). Furthermore, by contacting increasing amounts of TMAI with Eu-Cu, it was possible to obtain $Y_{P/B}$ increasing from 13.7 to 33.9 mg BSAE of TMAI per g of dry support and specific activities (π_{TB}) increasing from 1.9 to 5.4 IU/g. The catalytic efficiency of the immobilized enzyme was higher with the Eu-Cu supports as compared to Eu, suggesting that adsorption through His tags resulted in fewer conformational changes of the adsorbed protein, which could lower the enzyme activity.

Table 5. Immobilization of purified TMAI on 0.04 g (m_{Bd}) of Eu: V_0, C_{P0}, C_{Pf}, TMAI solution volume, initial and final concentration, respectively; R, percentage of immobilization; $Y_{P/B}$, immobilization yield; π_{TB}, specific activity; Y_T, catalytic efficiency. Activity experiments were run in duplicate. Values with the same letter were not significantly different (Fisher's method applied to the natural logarithm of π_{TB} or Y_T to stabilize variances, 90% confidence level). Protein concentrations were estimated by using the Bradford method.

Exp.	Support	V_0	I	C_{P0}	C_{Pf}	R	$Y_{P/B}$	π_{TB}	Y_T
		ml	M	mg/ml	Mg/ml	%	mg BSAE/g ds	IU/g ds	%
5	Eu	3	0.05	0.21	0.06	72.09	11.5	0.52±0.04[a]	28±2[a]
6	Eu	3	0.33	0.19	0.02	88.90	12.8	0.67±0.11[a]	33±5[a]
7	Eu	3	0.67	0.19	0.02	90.03	12.7	0.82±0.22[a]	40±11[a]

The use of the purified enzyme for immobilization on Eu yielded Y_T values which appeared independent of the ionic strength of the buffer used and equal to 33±6 % (exp. A-C, Table 5). Thus the use of a purified TMAI with Eu did not result in any increase in the catalytic efficiency compared to immobilizations on Eu-Cu.

Table 6. Immobilization of purified TMAI on 0.04 g (m_{Bd}) of Eu: V_0, C_{P0}, C_{Pf}, TMAI solution volume, initial and final concentration, respectively; R, percentage of immobilization; $Y_{P/B}$, immobilization yield. Activity experiments were run in duplicate. Protein concentrations were estimated indirectly from activities measurements.

Exp.	Support	V_0	I	C_{P0}	C_{Pf}	R	$Y_{P/B}$
		ml	M	mg/ml	mg/ml	%	mg BSAE/g ds
8	Eu-Cu	95	0.05	0.24	0.05	78.7	35.7
9	Eu-Mn	95	0.05	0.24	0.20	17.5	8.0
10	Eu-Cu	95	0.05	0.15	0.03	79.7	23.1
11	Eu-Cu	190	0.05	0.023	0.05	76.5	33.5

Two immobilization experiments were carried out to compare the immobilization capability of Eu-Cu and Eu-Mn supports. The results obtained (Exp. 8 and 9, Table 6) show that Eu-Mn supports are characterized by a low immobilization yield, of the same order of Eu supports (see Exp. 1, Table 4). The idea of using Eu-Mn supports for the immobilization of TMAI came from the fact that the ion Mn^{2+} is required for TMAI activity/stability and thus using a support chelated with Mn^{2+} instead of Cu^{2+} could have represented an advantage. The low $Y_{P/B}$ obtained could be due to low quantities of Mn^{2+} chelated with the support (in fact it is known that Mn^{2+} has a lower affinity for the IDA matrix compared to Cu^{2+}, Co^{2+} or Ni^{2+}) and/or to the low adsorption affinity of the His tags present on TMAI towards the Mn^{2+} chelated to the IDA support.

Finally, the last two immobilization experiments yielded immobilized biocatalysts with immobilization yields reported in Table 6 (Exp. 10 and 11). It can be noted that the obtained $Y_{P/B}$ are in line with the ones obtained previously (Table 4, Exp. 3 and 4).

5.9 Thermal stability assays

Under the conditions studied, the decay in enzyme activity, expressed as the ratio (R) of the measured activity, $\pi_{TB}(t)$ or $\pi_{TE}(t)$, at time t of heat treatment to the initial activity (π_{TB0} or π_{TE0}) was described by a first-order decay as follows:

$$R = \exp(-k_d\, t) \qquad (6)$$

where k_d is the deactivation rate constant at a given process temperature. Fitting of the experimental data through Eq. (6) allowed to estimate k_d values and the corresponding standard errors, as reported in Fig. 18.

Fig. 18 shows that for immobilized TMAI it was not possible to determine its deactivation behaviour at 80°C because the enzyme did not lose a measurably significant amount of activity under the conditions studied for approximately 5 h. At 90°C immobilized TMAI halved its original activity in about 1 h compared to 2 h and 0.3 h for free TMAI at 80°C and 90°C respectively. Such a stabilization effect, corresponding to a stabilization factor (ratio of first order deactivation constant for the free and immobilized enzyme) of about 7 at 90°C, may be attributed to a mild multipoint covalent immobilization which can occur with epoxy supports.

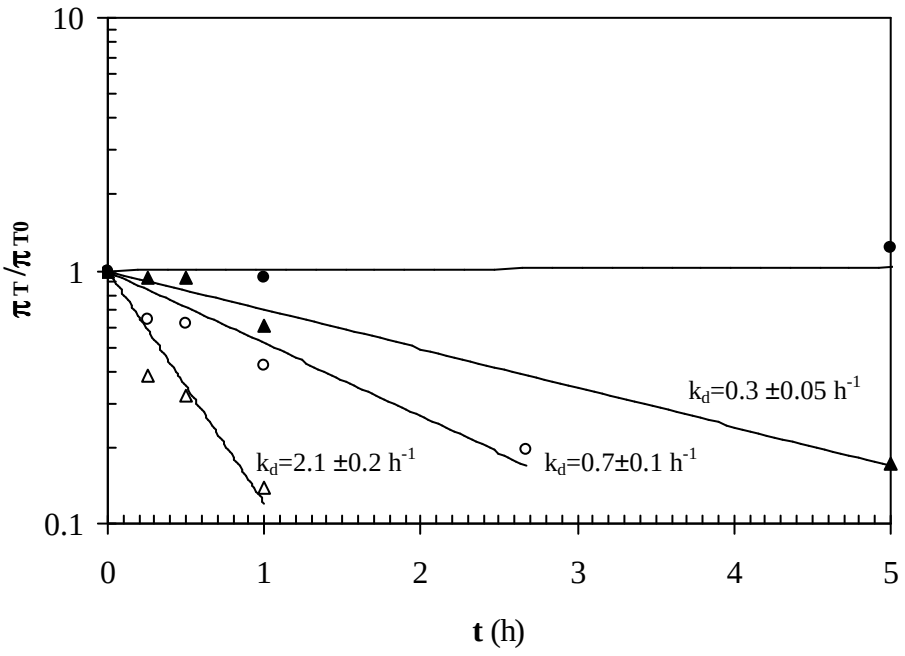

Figure 18. Time course of irreversible thermal inactivation of free (▲,△) and immobilized (●, ○) TMAI after various periods of incubation at 80°C (closed symbols) and 90°C (open symbols). Activity of heat treated samples were measured at 80°C with the standard reaction mixture.

5.10 Effect of temperature on immobilized TMAI activity

The activity of immobilized TMAI with the standard reaction mixture increased by about five times when temperature was increased from 60 to 80°C (Fig. 19). This is in line with the behaviour of thermophilic enzymes which generally double their activity for a temperature increase of 10°C.

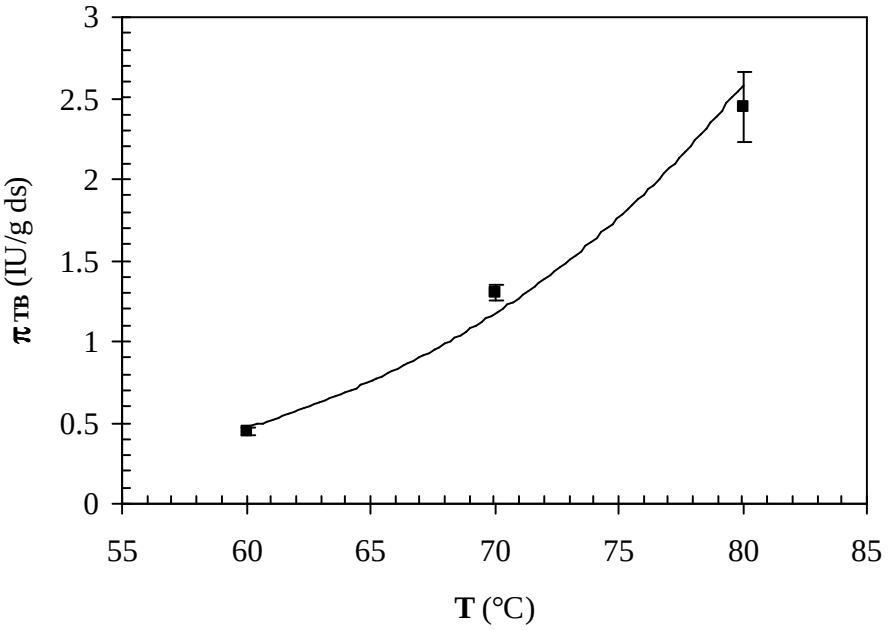

Figure 19. Effect of temperature on immobilized TMAI activity.

5.11 Effect of pH on immobilized TMAI activity

5.11.1 Initial activity

Immobilized TMAI had broad pH optima at 80°C (Fig. 20) and its specific activity changed little between pH 5 and 7.5.

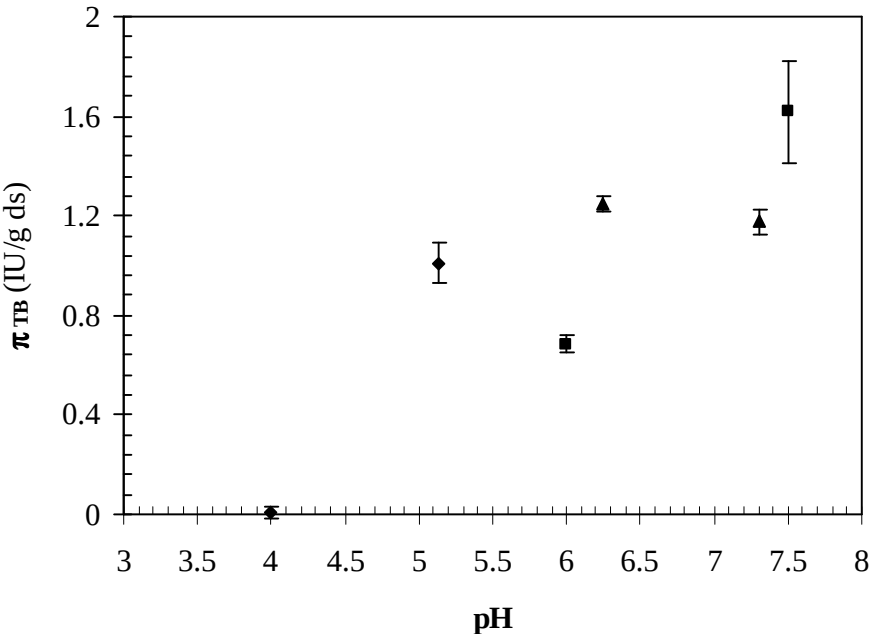

Figure 20. Effect of pH on immobilized TMAI activity at 80°C. The buffers used were sodium acetate (◆), sodium phosphate (▲) and Tris-HCl (■).

5.11.2 Activity over long incubation with different buffer systems and pH

In order to investigate the effect of the buffer system and pH on TMAI activity under operational conditions, reactions over long incubation times were studied. In activity measurements we always incubated reaction mixtures in presence and absence of the immobilized biocatalyst to study the non-enzymatic contribution to the isomerisation. This has not been described or stated explicitly in the literature, except for the work of Bandlish *et al.* (2002) in the case of the isomerisation of glucose to fructose. The absorbance readings determined by the cysteine-carbazole method for reaction mixtures incubated in absence of immobilized TMAI, were converted in terms of D-tagatose and are reported as a function of time in Fig. 20. It can be observed that with some buffers (phosphate, pH=7.3 and pH=6.25; tris-HCl, pH=7.5) the reaction mixtures developed a non-negligible colour with the cysteine-carbazole method. Analysis of this mixtures (see Fig. 22) for the phosphate buffer by HPLC identified the presence of D-tagatose and other non-identified peaks.

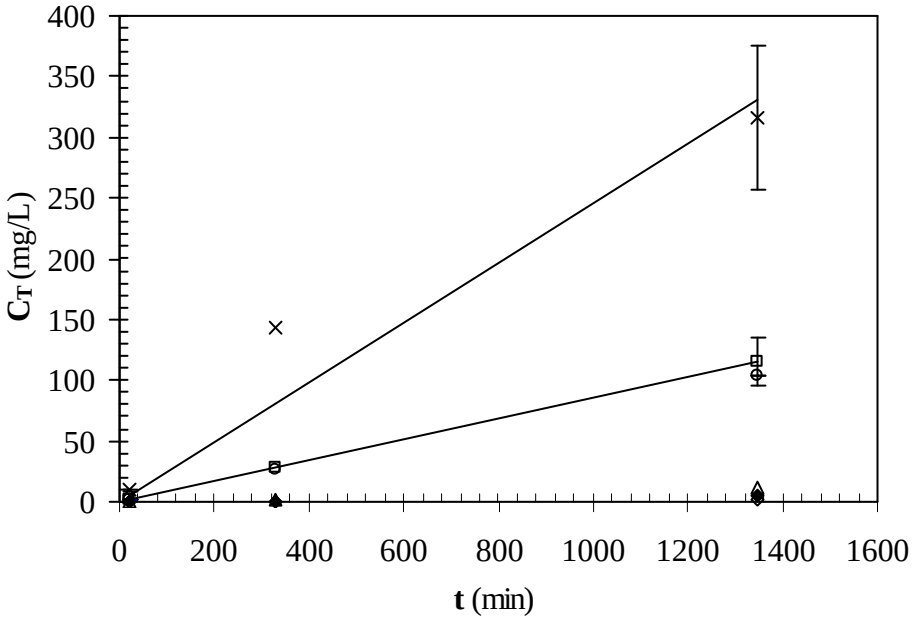

Figure 21. Colour development expressed as D-tagatose (C_T) obtained by the cysteine-carbazole method for reaction mixtures corresponding to different buffer systems and pH: phosphate buffer at pH 7.31 (x) or 6.25 (○); tris buffer at pH 7.5 (□) and 6 (●); acetate buffer at pH 5.14 (Δ) and 4 (◊). Each sample contained 4.5 g/l of D-galactose.

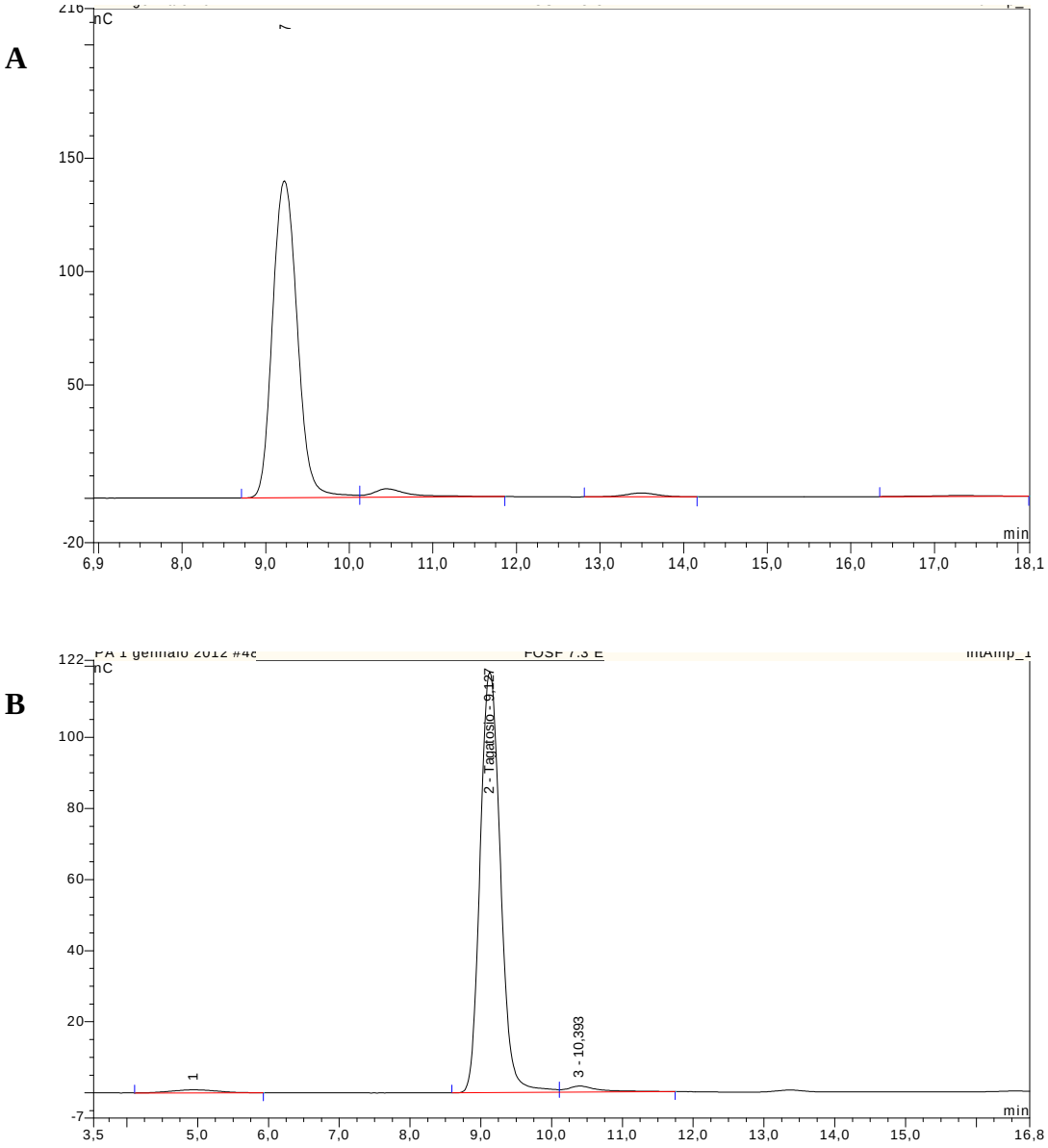

Figure 22. Chromatograms of a reaction mixture incubated in absence (A) or presence (B) of immobilized TMAI in phosphate buffer, pH 7.3.

Figure 23 reports the concentrations of D-tagatose measured by the cysteine-carbazole method for reaction mixtures incubated in presence or absence of the immobilized catalyst. It can be observed that the highest production of D-tagatose in presence of TMAI was obtained for tris-HCl buffer at pH 7.5. The chemical contribution to isomerisation was different for the different buffers used. In particular, the fact that D-tagatose production for the phosphate buffer at pH 7.31 was higher in absence of TMAI than in its presence seemed apparently strange.

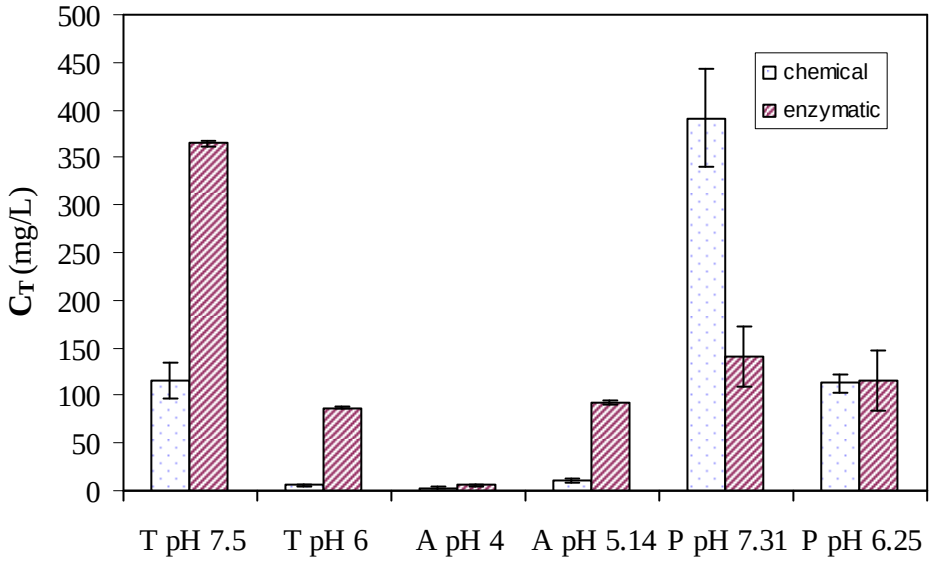

Figure 23. Effect of buffer system and pH on D-tagatose production in presence (enzymatic) or absence (chemical) of immobilized TMAI at 80°C for long incubation times (1345 min). The buffers used were Tris-HCl (T), sodium acetate (A) or sodium phosphate (P) at several pH values.

We hypothesized that in each system the support used for immobilization adsorbed or sequestered part of D-tagatose, subtracting it from the liquid medium. Thus the final D-tagatose observed resulted from the difference between production by TMAI and sequestration by the support. Based on these results we decided to use tris-HCl buffer at pH 7.5 for subsequent studies and discard phosphate buffer. The lower activity observed with phosphate buffer in long incubation experiments may be attributed to the fact that when $MnCl_2$ is added to the buffer a precipitate is formed, probably manganese phosphate, thus depriving the reaction medium of Mn^{2+} which is important for the stability of TMAI (Lee *et al.*, 2004).

5.12 Post-immobilization stabilization treatments

The post-immobilization stabilization treatments did not affect appreciably the initial activity of the Eu-Cu immobilized derivatives, as can be seen from Figure 24. Furthermore, there was not a decrease of the initial activity of stabilized derivatives (A-D) compared to the non-stabilized one (E). In general, an effective stabilization treatment can produce a decrease of the initial activity but such an activity decline should be compensated with an enhancement in the stability of the enzyme. The activity of the stabilized derivatives was practically nil when the buffer did not contain Mn^{2+} or contained Mg^{2+} instead of Mn^{2+}.

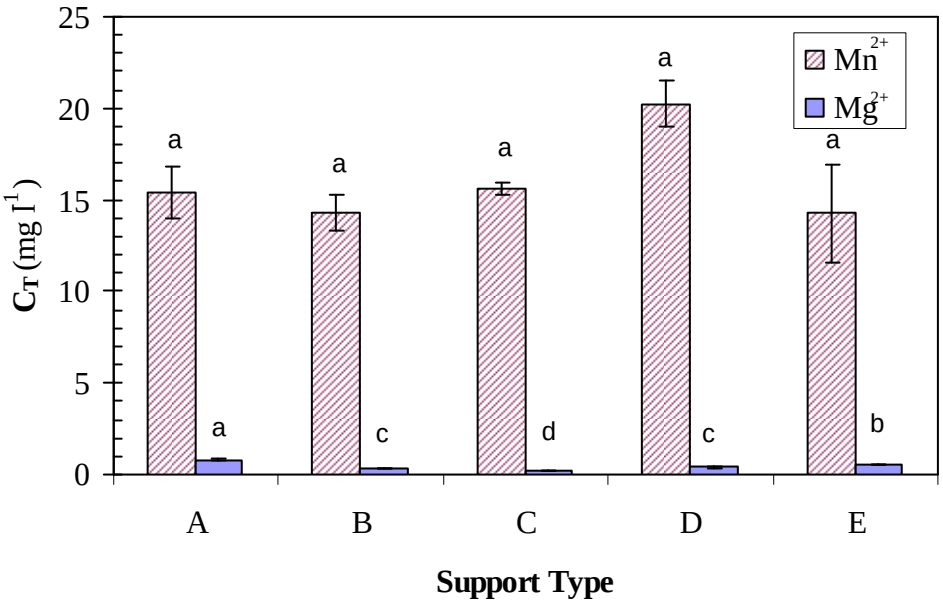

Figure 24. Results of activity measurements of Eu-Cu supports subjected to different post-immobilization stabilization treatments: concentration of D-tagatose (C_T) obtained after 20 min of incubation at 80°C in presence of Mn^{2+} or Mg^{2+}. Treatments of the same series (Mn^{2+} or Mg^{2+}) with the same letter do not differ significantly (α=5%, Fisher's LSD method).

The method of post-immobilization stabilization affected the activity of the biocatalyst over long incubation times and thus its operational stability (Figure 25). The treatment with EDA and glutaraldehyde (A) resulted the best, the stabilized derivatives producing an almost double D-tagatose quantity compared to the other treatments and to the non-stabilized derivatives (E).

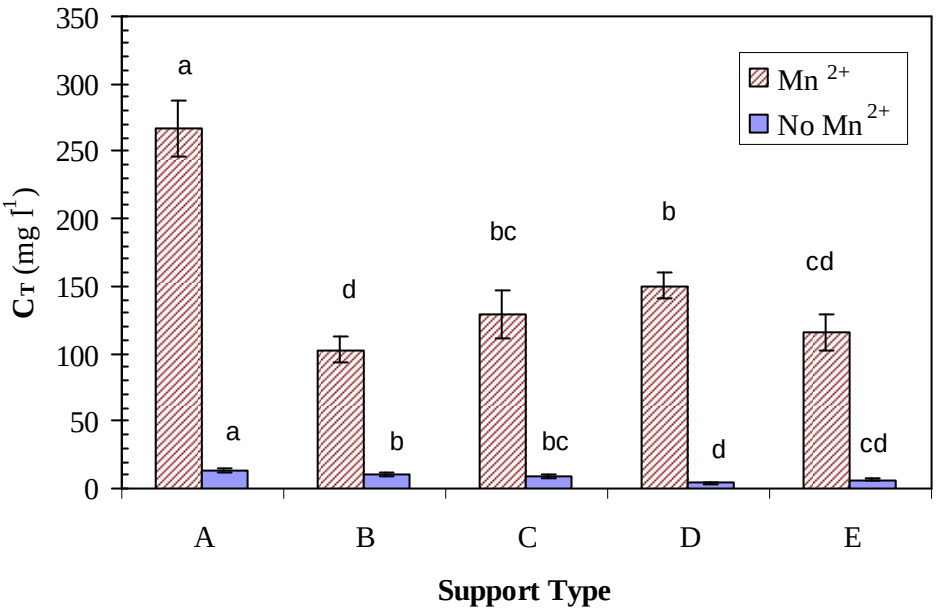

Figure 25. Results of activity measurements of Eu-Cu supports subjected to different post-immobilization stabilization treatments: concentration of D-tagatose (C_T) obtained after 860 min of incubation at 80°C in presence or absence of Mn^{2+}. Treatments within each group (Mn^{2+} or No Mn^{2+}) with the same letter do not differ significantly (α=5%, Fisher's LSD method).

5.13 SDS-PAGE analysis of the multi-subunit stabilization of immobilized TMAI

Fig. 26 shows the SDS-PAGE analysis of supernatants from the five immobilized derivatives subjected to the stabilization treatment (A-E), heat-treated in presence of SDS and 2-mercaptoethanol. Several *E. coli* proteins along with TMAI subunit, corresponding to the band at about 55 kDa, can be observed. It is clear that the stabilization treatment with EDA and glutaraldehyde (treatment labelled as A) was able to bind covalently all the TMAI monomers to the support, directly or indirectly, as well as all the other proteins immobilized on the support. Thus, the higher stability of the A support was also associated with a multi-subunit stabilization/immobilization. Treatments B, C and E did not seem to stabilize the multi-subunit structure of TMAI. For the treatment with glutaraldehyde (D), it was not possible to obtain a clear SDS-PAGE gel. This may be due to the fact that the proteins released from the support bound at the top of the gel, at the beginning of the run or that some proteins are crosslinked by glutaraldehyde and are released as an about 200-kDa molecular weight aggregate. Thus, it is not clear if treatment D was effective in stabilizing the multi-subunit nature of TMAI.

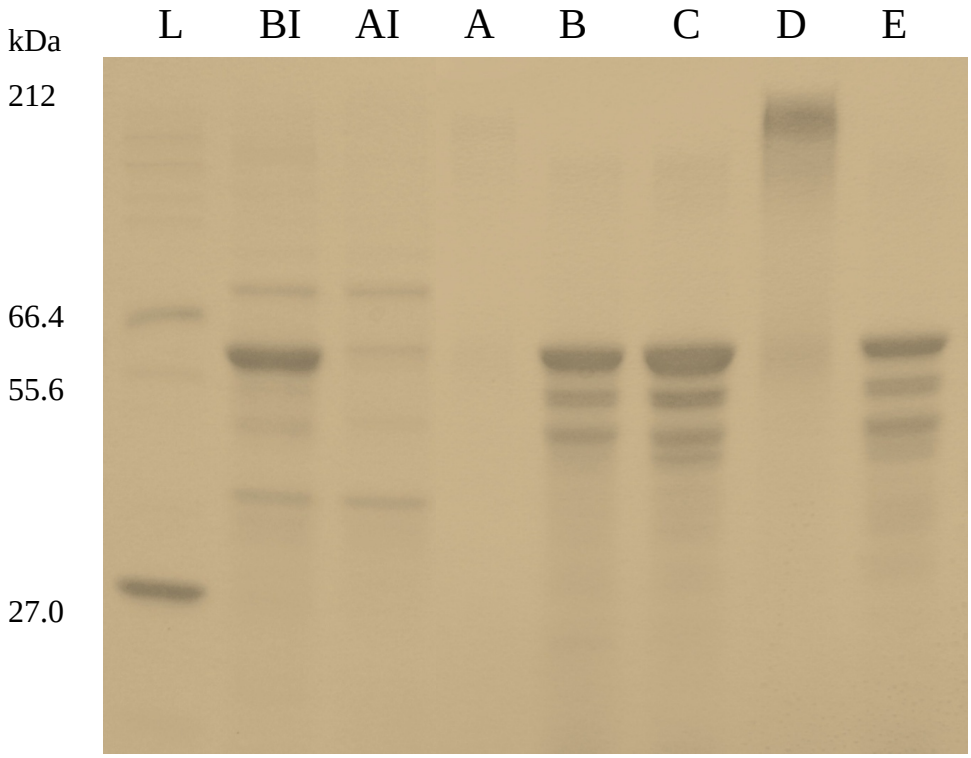

Figure 26. SDS-PAGE analyses of supernatants obtained after boiling the immobilization solution at the beginning and at the end of immobilization and five different derivatives in the presence of SDS as described in the Materials and Methods section. Lane L, molecular mass marker; lane BI and AI, immobilization solution at the beginning and at the end of immobilization, respectively; lane A, B, C, D, E, immobilized enzyme derivatives stabilized under different conditions (see section 4.11).

The unsuitability of Eupergit ® C 250 L under the post-immobilization treatment protocols B, C and E to stabilize the quaternary structure of TMAI and, consequently, to improve its thermal stability shows that it is difficult to link covalently all the subunits of a multimeric enzyme on the support internal surface. In particular, the pore diameter (180-300 nm) of Eupergit ® C 250 L is much larger than the diameter of TMAI (~ 8 nm), that can be estimated starting from its molecular mass as the minimum diameter of a smooth sphere containing the whole protein (Erickson, 2009). Thus, each molecule of TMAI should see the support internal surface as a plane and this could make it geometrically impossible for all the enzyme subunits to establish a connection with the support even when several nucleophiles on each subunit are available to react with the un-reacted oxirane groups.

The effect of the internal support morphology was also described by Fernández-Lafuente *et al.* (1999) who immobilized *E. coli* extracts on highly activated amino-supports, such as Toyopearl support formed by very thin fibres with approximately the same protein

dimensions, and Sepabeads support having a large internal surface with pore size of about 20 nm. While after the heat treatment Sepabeads derivatives released only some protein subunits, Toyopearl derivatives freed many proteins, thus signifying that the morphology of the support surface plays a role in the stabilization of the quaternary structure of proteins. The difficulty of stabilizing the quaternary structure of a complex multimeric enzyme immobilized on an epoxy support under conditions that should favour multipoint covalent attachment was observed also by Bortone *et al.* (2012) with the enzyme urease immobilized on Eupergit® C250L (see section 3.5.3 for more details).

5.14 Effect of mercaptoethanol treatment on immobilized/stabilized derivatives.

The activity over long times of derivatives treated with mercaptoethanol was higher compared to non-treated derivatives. Figure 26 shows the results obtained for non-stabilized derivatives. At short times, the produced D-tagatose was not affected by mercaptoethanol treatment while at long times there was a marked improvement. A similar behaviour was observed for derivatives stabilized by EDA and glutaraldehyde (labelled as A). It can be observed that at long times, when using derivatives not treated with mercaptoethanol, D-tagatose concentration reached a plateau or started to decrease after a maximum, while with derivatives treated with mercaptoethanol its concentration continued to increase even if at a smaller rate compared to its rate at short times. Thus, treatment of immobilized derivatives with mercaptoethanol appeared to improve considerably D-tagatose production.

It has been reported that mercaptoethanol is able to release all Cu^{2+} ions from the Cu-Eu supports (Mateo *et al.*, 2000b). A possible explanation of the reduced activity when using derivatives not treated with mercaptoethanol is that the Cu^{2+} gradually released from the support affects negatively the activity of the enzyme at a point that the activity is fully stopped. In order to validate this hypothesis we performed activity experiments with the free enzyme incubated with Mn^{2+} in absence or presence of Cu^{2+} and observed no D-tagatose production in the latter case (data not shown). Since mercaptoethanol is used as an alternative to glycine to block unreacted epoxy groups present on the support, we suggest the use of mercaptoethanol instead of glycine for the immobilization of metal-chelate epoxy supports.

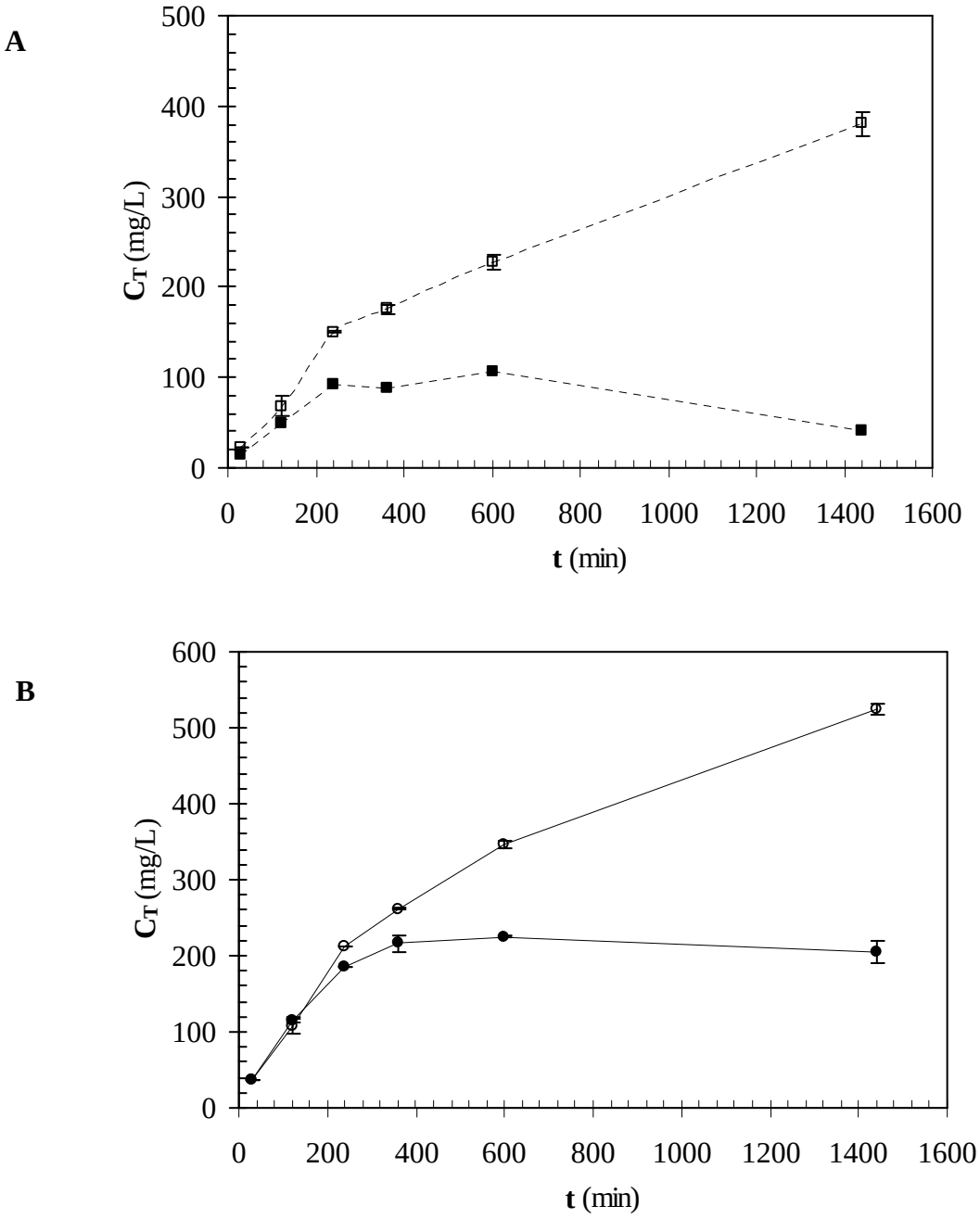

Figure 27. Concentration of D-tagatose (C_T) vs. time (t) in long incubation experiments for derivatives treated (open symbols) or not (closed symbols) with mercaptoethanol. Data refer to non stabilized derivatives (A, □,■) or to stabilized derivatives (B, ○,●) obtained under the condition A (derivatives stabilized with EDA and glutaraldehyde).

5.15 Repeated-batch bioconversion experiments

The possibility of using immobilized TMAI at 60°C for repeated bioconversion experiments was checked at 4.5 or 18 g/l initial D-galactose concentration. Fig. 27 shows the time course of D-tagatose production for three cycles. It can be seen that the biocatalyst did not lose appreciably its activity after 242 h of operation at 60°C. D-galactose conversion (X_G, defined

as the ratio between the mass of consumed D-galactose and its initial mass) and average productivity were estimated for the second cycle as 44.4 % and 0.02 g/l h or 29.1% and 0.06 g/l h at 4.5 g/l or 18 g/l initial D-galactose concentration, respectively.

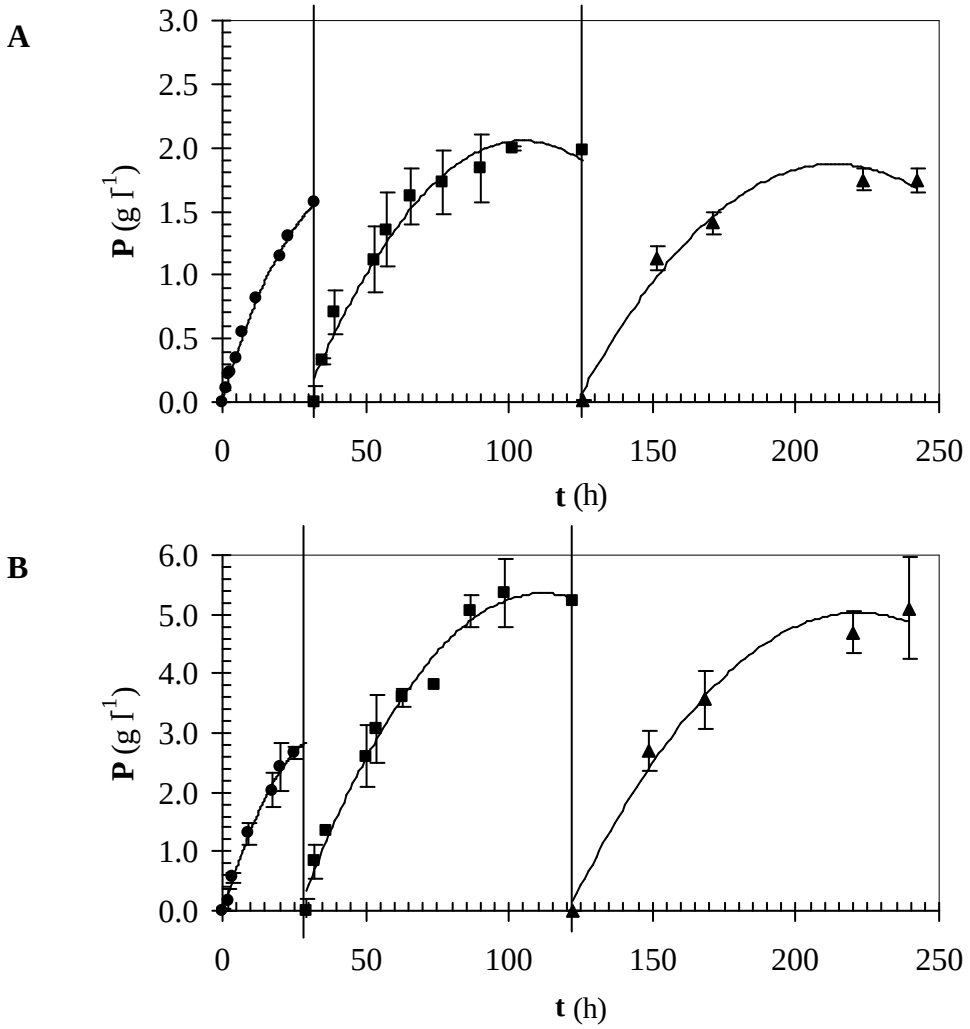

Figure 28. Production of D-tagatose at 60°C and 4.5 (A) or 18 g/l of initial D-galactose concentration (B) in repeated batch experiments: concentration of D-tagatose (P) vs. time (t) (●, first cycle; ■, second cycle; ▲, third cycle).

The maximum conversion of D-galactose (X_G) that can be produced in the isomerisation reaction corresponds to the equilibrium conditions and is linked to the chemical equilibrium constant (K) by the following equation:

$$X_G=K/(K+1) \qquad (7)$$

where K is a function of temperature only. An approximate value of K equal to 1 at 60°C can be obtained from the data of Figure 8, yielding a maximum D-galactose conversion at 60°C of 50%. The batch bioconversion experiments at 60°C yielded conversions of 44.4% and 29.1% for initial D-galactose concentrations of 4.5 and 18 g/l, respectively. It is not clear why the bioconversion seemed to stop at conversions lower than the equilibrium one but this behaviour has already been reported in the literature. Lim *et al.* (2008) investigated the production of D-tagatose at 70°C and pH 7.5 in a stirred tank reactor containing immobilized L-arabinose isomerase from *Thermotoga neapolitana*. The conversion attained decreased from 38% to 20% when the initial concentration of D-galactose increased from 100 to 500 g/l. The authors attributed this result to the pH change during the reaction. The corresponding conversions obtained with pH control were 53 and 38%, still lower than the equilibrium conversion at 70°C corresponding to about 58%. In this work we measured the initial and the final pH of the solution and we did not observe any appreciable change.

The possibility of using immobilized TMAI at 80°C for repeated bioconversion experiments was checked at initial D-galactose concentrations of 18 g/l. Figure 29 shows the time course of D-tagatose production for three cycles for two different immobilized biocatalysts. A direct comparison between the results presented in Figure 29A and 29B cannot be done since the initial activity and the quantity of the dry weight of the biocatalyst used was different. It is however clear that the biocatalyst stabilized and treated with mercaptoethanol (Figure 29B) decreased its activity at a slower rate during the three cycles compared to the biocatalyst that was neither stabilized nor treated with mercaptoethanol. In fact the change in the productivity from the first cycle to the third one was from 0.40 to 0.09 g/l h for the untreated biocatalyst and from 0.41 to 0.34 g/l h for the one stabilized and treated with mercaptoethanol. Furthermore, the experiments carried out with the biocatalyst non stabilized (Fig. 29A) show that for each cycle a plateau concentration is approached which is different from the one corresponding to equilibrium (2.9-13.9% against 66%). This behaviour is consistent with the repeated batch experiments carried out at 60°C and with the long time incubation experiments carried out at 80°C (see Fig. 27A and 27B). The treatment with mercaptoethanol seems to avoid this problem, since both long incubation experiments and repeated batch experiments at 80°C (Fig. 29B) are characterized by a continuous increase of D-tagatose.

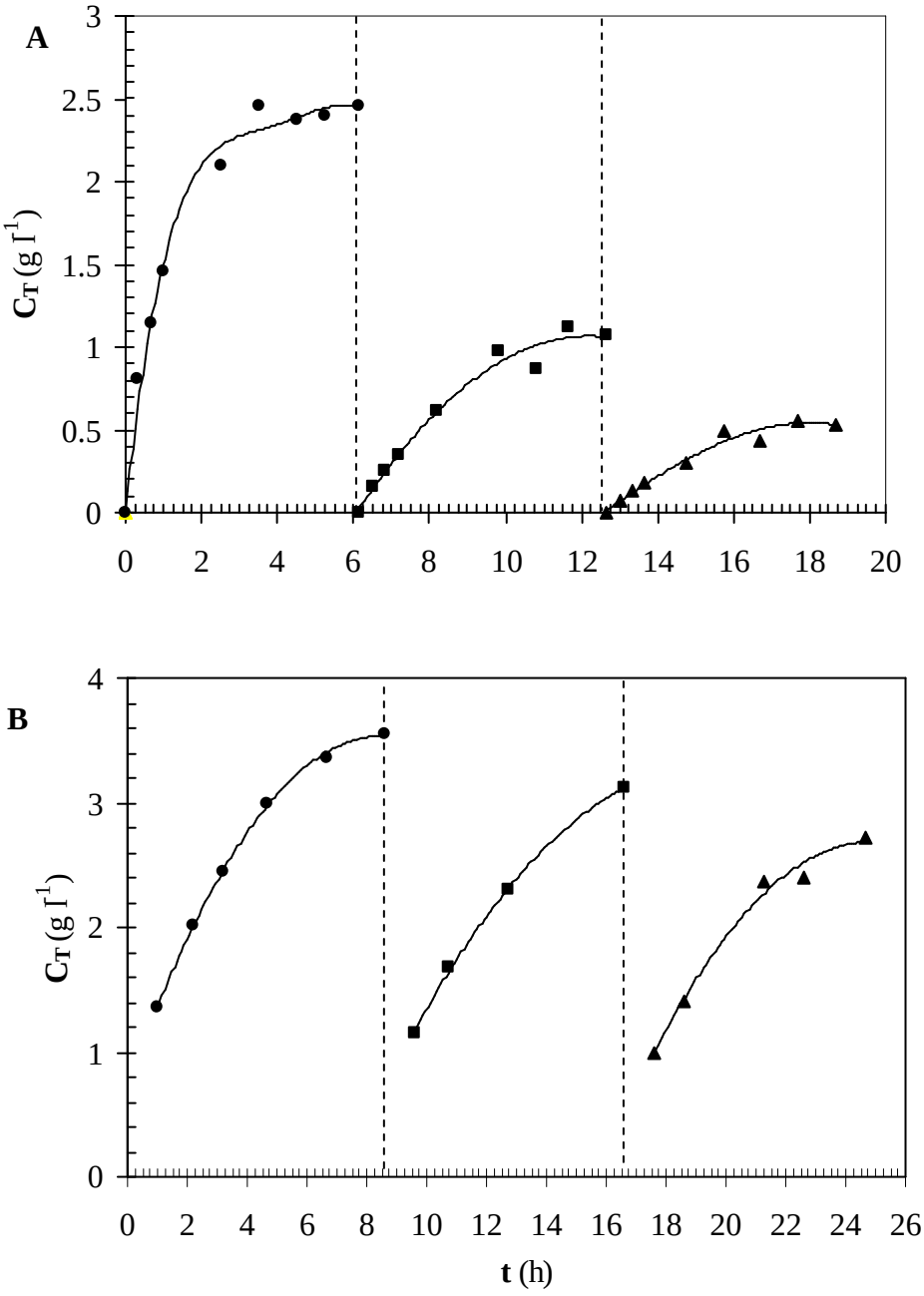

Figure 29. Concentration of D-tagatose (C_T) at 80°C and 18 g/l of initial D-galactose concentration vs. time (t) in repeated batch experiments: A, non-stabilized immobilized derivatives (treatment E); B, stabilized immobilized derivatives (treatment A) treated with mercaptoethanol (●, first cycle; ■, second cycle; ▲, third cycle).

6. Conclusions

His-tagged L-arabinose isomerase from *Thermotoga maritima* (TMAI) was immobilized selectively on copper-chelate Eupergit C250L. Starting from a partial purified preparation, it was possible to obtain high immobilization yields of 33 mg BSE /g ds with a high catalytic efficiency of almost 100%. While it has been reported that Mn^{2+} and/or Co^{2+} ions are required for the stability of TMAI, in view of its potential application in the production of food-grade D-tagatose, only the Mn^{2+} ion at a concentration of 5 mM was used in this work to characterized the catalytic properties of the immobilized enzyme. The maximum specific activity of the immobilized biocatalyst at 80°C, pH 7.5 and 4.5 g/l D-galactose obtained in initial rate measurements was equal to 5.4±1.1 IU/g ds. When kept at 80°C in absence of D-galactose the biocatalyst was stable for more than x hours. However, when incubated under operative conditions with D-galactose a 18 g/l and at 80°C, the activity vanished after 3 cycles of 6 hours each. This results suggest that at 80°C the deactivation of the enzyme in presence or absence of D-galactose is different, most likely because when the sugar is present several side reactions could occur leading to the deactivation of the enzyme. Furthermore, repeated batch isomerisation experiments at 60°C and 80°C showed that a plateau conversion is obtained in each cycle that is lower than the one corresponding to chemical equilibrium, that is 50% and 66%, respectively. The effect of several post-immobilization treatments on the immobilized-biocatalyst activity under operational conditions was studied. It was found that a post-immobilization treatment with EDA and glutaraldehyde stabilized the multi-subunit nature of the enzyme (there was no leakage of enzyme monomers from the immobilized derivatives), a desirable property for enzymes to be used in food applications. Furthermore,

this type of stabilization was accompanied by an increase in D-tagatose production under operative conditions compared to the non-treated biocatalysts most likely because of an increase in thermal stability. Immobilized derivatives as such or stabilized with EDA and glutaraldehyde were subjected to a further post-immobilization treatment with β-mercaptoethanol. β-mercaptoethanol can reduce the Cu^{2+} ion and thus facilitate its release from the matrix. Experiments with both stabilized and not stabilized derivatives aimed at estimating the effect of β-mercaptoethanol showed that when the biocatalyst had not been treated with β-mercaptoethanol, the concentration of D-tagatose reached a plateau while it continued to grow when not treated with β-mercaptoethanol. This finding suggests that Cu^{2+} interferes negatively with the enzyme activity. A final experiment was carried out with the biocatalyst stabilized and treated with β-mercaptoethanol in repeated batch isomerisations carried out at 80°C in a packed-bed column. D-tagatose concentration grew continuously during each cycle and the biocatalyst did not lose appreciable activity after three cycles of 8 hours each. While further studies are required to investigate the use of TMAI for the industrial production of D-tagatose, this study showed that the combined use of selective adsorption of IMAC (immobilized metal affinity chromatography) toward His-tagged L-arabinose isomerase with the properties of Eupergit C250L allowing for covalent immobilization and post-immobilization treatments can result in immobilized enzymes with improved operational stability and performances.

7. References

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