# IMMOBILIZED ENZYMES: LACTASE BONDED TO STAINLESS STEEL AND OTHER DENSE CARRIERS FOR USE IN FLUIDIZED BED REACTORS

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Lactase has been attached to stainless steel and other dense carriers for use in fluidized bed reactors. The support is activated by coating it with a layer of titanium oxide. Heating the activated support at 538° before contacting with enzyme solution seems to improve catalytic activity and stability of supported lactase. Optimum pH of free and supported enzyme was 4.5. Optimum temperature for free enzyme was 60°; for steel-supported lactase, it was 70°. Heat treated steel-lactase adduct retained 44.5% of initial activity after 5 weeks at 45° under water. The bonding method has also been applied successfully to α-amylase, amyloglucosidase, asparaginase, catalase and trypsin. Inactivated catalyst can be regenerated by heating to 538°, cooling and recontacting with enzyme solution. This is believed to be the first report of enzymes bonded to stainless steel.

The presence of lactose in milk and milk by-products is undesirable for physiological (1) and economic (2) reasons. The use of immobilized derivatives of lactase has been suggested for removing this sugar from dairy products (2-6). Charles, Coughlin and coworkers (7,8,9) have successfully used lactase immobilized to steel, nickel oxide and alumina particles in fluidized-bed reactors for processing cheese whey; this paper reports in detail on the immobilization techniques. The most favorable operation of such reactors requires that the enzyme be attached to particles of relatively high density. Stainless steel was chosen not only for its high density, but also because of its recognized inertness and widespread acceptance for vessels used in food processing. Fine particles (100-200μ) of stainless steel were activated by

coating with titanium oxide formed by the hydrolysis of $TiCl_4$; this is different from the technique of Barker et al (10) who contacted glass, cellulose and nylon directly with $TiCl_4$; we have also found that lactase and other enzymes readily bond to the activated steel. Commercially available sintered nickel oxide (177-420μ) and alumina cracking catalyst (177-420μ) were also found to be suitable enzyme supports which could be activated by this technique.

Materials and Methods: Lactase LP, derived from Aspergillus niger and purchased from Wallerstein Laboratories; $TiCl_4$, purchased from Fisher Scientific Co. and α-lactose, purchased from Sigma Chemical Co., were used as received. Stainless steel from Glidden Metals Co. was sieved to get -100+150 mesh particles. Nickel oxide "Sinter 90" from International Nickel Co. and alumina carrier Type SAEHS-33 (Carborundum Co.) were sieved to get -40+80 mesh particles. The nickel oxide is believed to be non-porous. Alumina had a surface area of 4.0 $m^2/g$, a porosity of 0.32 cc/g and a bulk density of 1.2 g/ml.

One hundred grams of stainless steel and 300 ml deionized water in a 1 liter 3 neck, round bottom flask fitted with a mechanical stirrer, a dropping funnel and a gas evolution tube, were cooled to 5-10°C in a ice-water bath. Fifty ml $TiCl_4$ were added to the constantly stirred suspension over a period of 5-10 minutes. The mixture was stirred, in the ice bath, an additional 30 minutes after the addition was completed. The ice bath was removed and stirring was continued for another 30 minutes at ambient temperature. Excess solution was decanted and the activated steel was freed from non-adhering particles of Ti oxides by elutriation with water. Half of the steel was air dried at room temperature. The remaining material was heated at 538° for 1 hour. It was elutriated with

TABLE 1

| Sample | Mg.protein/ gram of adduct | Lactase units/ gram of adduct | Mg.protein/ ml mother liquor | Lactase unit: ml mother liquor |
|---|---|---|---|---|
| Stainless steel | | | | |
| Non-activated | 3.65 | 163 | 9.4 | 777 |
| Activated | 16.12 | 1208 | 8.0 | 736 |
| Activated;heated | 17.14 | 1405 | 7.4 | 780 |
| Alumina | | | | |
| Non-activated | 6.33 | 1748 | 7.3 | 1919 |
| Activated | 10.63 | 2440 | 4.5 | 242 |
| Activated;heated | 13.82 | 2742 | 1.0 | 283 |

Approximately 0.2 grams of supported enzyme or 0.1 ml of mother liquor was added to a solution, pre-warmed to 37°, containing 10 ml 20% lactose in citrate phosphate buffer, pH 4.5, and 1.5 ml of the buffer. Materials were agitated in a shaker bath at 37° for 20 minutes. Two ml of solution were withdrawn and added to 8 ml deionized water in a boiling water bath. Boiling was continued another five minutes. Glucose produced was determined by the Glucostat method. Enzyme and substrate blanks were nil. After incubation, supported enzymes were washed with water and dried at 130°. Dry weights were used in all calculations.

Citrate phosphate buffer is prepared using 0.1M citric acid and 0.2 M $Na_2HPO_4$.

One lactase unit is the amount of enzyme required to hydrolyze $3.16 \times 10^{-8}$ moles of lactose per minute under conditions of the assay.

Calculations:

$$\frac{\text{Lactose units}}{\text{gram}} = \frac{(\text{mg glucose/ml})(11.5\ \text{ml})}{20(1.08 \times 10^{-2})(\text{dry weight})} = \frac{101\ (\text{mg glucose/ml.})}{\text{dry weight}}$$

Free enzyme activity: 200 - 280 Lactase units/mg.

water and heated at 538° for another 30 minutes. After a final aqueous elutriation, the steel was air dried at room temperature. Nickel oxide and alumina were activated by the same procedure. Two gram samples of the activated steel and the heat-treated activated steel were weighed into 50 ml Erlenmeyer flasks. Fifty mg. of lactase and 3 ml citrate-phosphate buffer, pH 4.5 were added to each. A 2 gram sample of unactivated stainless steel was treated identically. All samples were agitated at 5°C in a

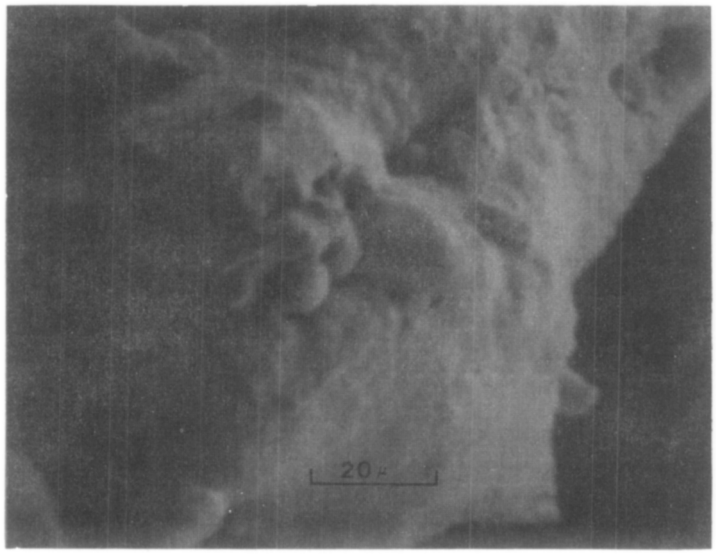


Fig. 1 Untreated ASTM 316 stainless steel particle

Figs. 1-5 show scanning electron micrographs taken on an ARL scanning electron microprobe in the secondary electron mode of operation. All photo magnification powers are 1050X. Pore sizes on Figure 2 range from approximately 4μ to 0.5μ. Other pore sizes can be estimated by comparison.

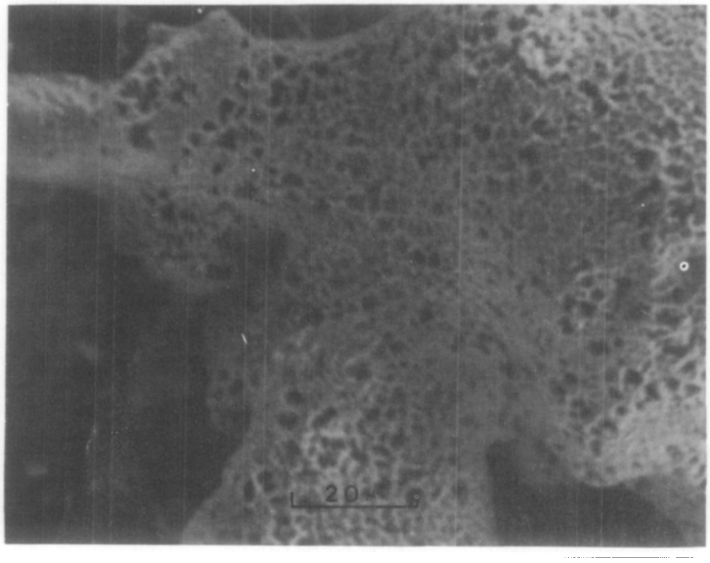


Fig. 2 Ti $C\ell_4$ activated 316 stainless steel. Entire surface is coated with $TiO_2$.

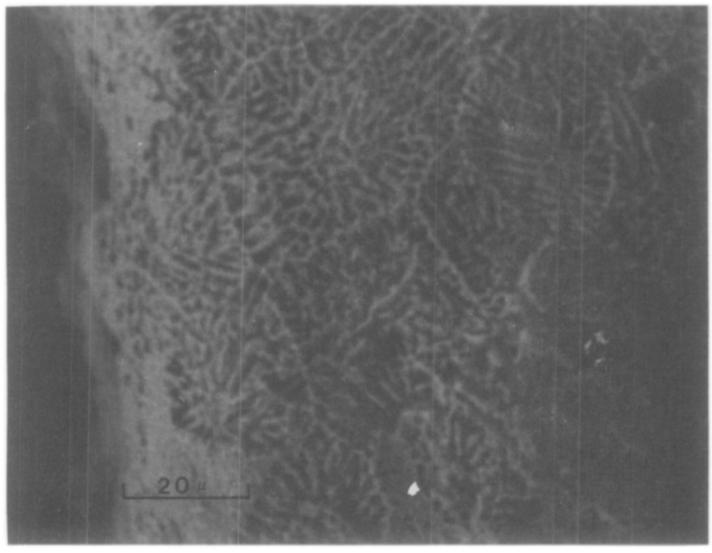


Fig. 3 Heat treated, Ti $C\ell_4$ activated 316 stainless steel. Entire surface is coated with $TiO_2$ and pore size is increased.

refrigerated shaker bath for at least sixteen hours. After this reaction period, the mother liquors were removed and saved for activity and protein concentration determinations. The steel-lactase samples were washed at least four times with deionized water. Enzymic activities were determined and protein contents were estimated by the Lowry method (11) using bovine serum albumin as standard. Data are reported in Table 1.

Results and Discussion: It is generally recognized that stainless steel has oxide and hydroxide groups on the surface and the existence of hydroxyl groups on the surface metal oxides is well established (12,13). The work of Hider and Marchessault suggests that $TiCl_4$ reacts with all oxygen functions (14). Barker postulated that $TiCl_4$ activated matrices bind enzymes by chelation (10). Thus, it might be surmised that $TiCl_4$ reacts with oxygen functions on the

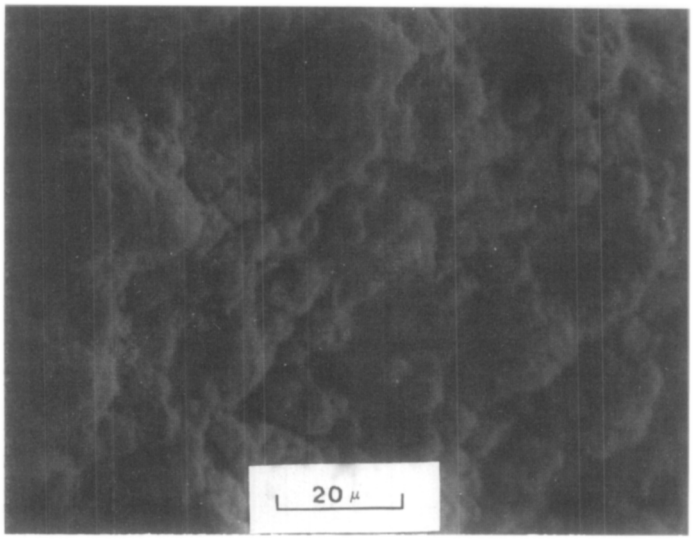


Fig. 4 Untreated Ni O particle

steel surface and binds the enzyme by chelation. We have achieved better results however by using a method in which the $TiCl_4$ is introduced into a hydrolyzing environment, under which conditions a porous layer of titanium oxide appears to form on the surface of the carrier particles; enzymes are then bound to this porous layer by sorption. The altered exterior surface of carrier particles so treated is revealed by scanning electron micrographs (Figs. 1-5) which suggest major changes in the carrier surfaces and deposition of a titanium oxide layer as a result of the activation treatment. BET measurements indicate that this activation increases specific surface area by about a factor of 10.

Optimum pH for all preparations, after correcting for acid hydrolysis of lactase, was 4.5. Optimum temperature for free enzyme was 60°; for steel-enzyme adduct, it was 70°. Both free and supported lactase lost most activity after 24 hours at 60°. The enzyme supported on heat-treated activated steel retained 44.5% of initial activity after 5 weeks when stored under de-

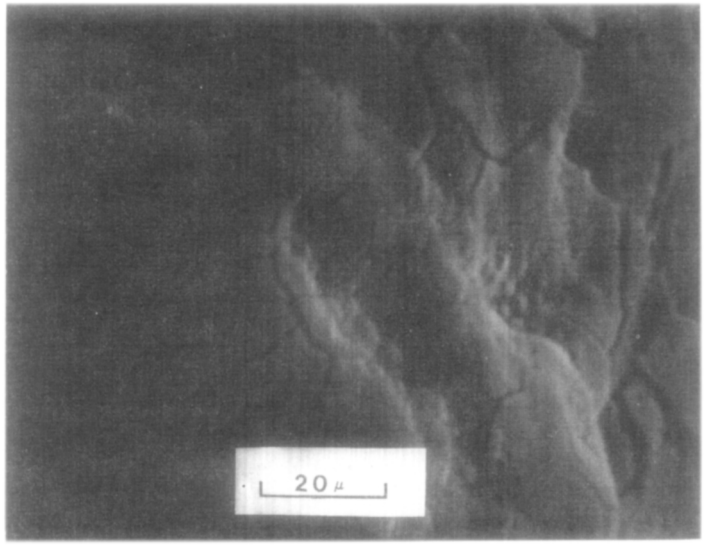


Fig. 5 Ni O treated with Ti $C\ell_4$ producing a layer of plate-like structures on the surface.

ionized water at 45°, while the non-heat-treated adduct retained 37.5% of original activity.

A fixed bed column packed with NiO supported lactase having an initial activity of 1010 LU/g, operated at 25°C and pH 3.5, using a flow rate of 1 ml/minute effected 100% conversion of a 1% lactose solution for 80 days. After another 40 days, the conversion was 92%. A similar catalyst was regenerated by heating in air for 0.5 hour at 538°C, (thereby burning off organic material) and re-contacting with fresh enzyme solution.

Some advantages of inorganic supports have been discussed by Weetall (15) and reviewed by Weetall and Messing (16). Advantages of pellicular heterogeneous catalysts have been described by Horvath and Engasser (17). An added advantage of the pellicular enzymic catalysts described here by us is the high density which permits the use of very small particles even at relatively high flow rates in fluidized bed reactors.

Data reported in Table 1 for steel supported samples are best data. More typical results are 20-30% of these values. Data reported for Ni O and alumina carriers are typical.

Although it has been claimed that the Corning method has been applied to materials ranging from glass to stainless steel (15), no data was presented, and this is believed to be the first report of enzymes attached to stainless steel. The binding method has also been applied to α-amylase, amyloglucosidase, asparaginase, catalase and trypsin. Further characterization of the catalysts is in progress and engineering data continues to accumulate.

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