UNIVERSITÉ DU QUÉBEC À TROIS - RIVIÈRES.

RÉSUMÉ SUBSTANTIEL DE MÉMOIRE EN MAÎTRISE DES SCIENCES DE L' ENVIRONNEMENT

PAR

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# INFLUENCE DE LA COMPOSITION SALINE SUR LA BIOVALORISATION DE L'EFFLUENT DE FROMAGERIE.

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## TABLEAU DE MATIÈRE

# I INTRODUCTION

La production du fromage au Québec et au Canada est en croissance régulière, elle a atteint 159.5 tonnes métrique (TM) et 370.2 TM en 1994 respectivement. On a estimé que 2,203 TM du lactosérum (petit lait) furent produits au Canada en 1994[(1)] . Le lait entier est utilisé pour la production du fromage naturel tel que le cheddar et le sous - produit résultant est appelé lactosérum doux, ayant un pH de 5 - 7. Le lactosérum est aussi le sous - produit de fromage lorsque les matières grasses et la caséine sont enlevées. Il est généré dans une proportion de 9 kg de lactosérum pour chaque kg du fromage fabriqué. Le fromage cottage, fabriqué avec lait écrémé, produit un lactosérum acide, pH de 3.5 - 5 dans une proportion d'environ 6 kg de lactosérum pour chaque kg du fromage fabriqué. Cette baisse de pH est due aux acides produits et / ou utilisés pour la coagulation. Le lactosérum est très riche en minéraux : Calcium, Sodium, Potassium, cuivre et fer. Ces minéraux ont une qualité digestive supérieure. Le lactosérum est également une source potentielle de complexes de vitamine B, de la riboflavine et d'autres vitamines.

L'utilisation du lactosérum est limitée à cause du lactose, son composant principal qui se cristallise facilement. La biovalorisation des effluents de fromagerie (lactosérum) et la récupération des sous-produits de biotransformation offrent des potentiels d'applications innombrables. Ces techniques pourraient

résoudre les problèmes de l'intolérance au lactose, de la cristallisation du lactose, et les problèmes environnementaux (pollution) associés au lactosérum.

Beaucoup de recherches ont été effectuées pendant les deux dernières décennies dans le domaine de valorisation du lactose, surtout la biotechnologie. Plusieurs chercheurs[2,3,4] ont mis l'accent sur l'hydrolyse du lactose par l'enzyme β - galactosidase. Quelques uns ont montré que les sels ou ions métalliques (cations) jouent un rôle important sur l'activité et la stabilité de la l'enzyme β - galactosidase pendant l'hydrolyse du lactose. Ces cations sont impliqués dans certains contrôles physiologiques, ils déclenchent les mécanismes d'activation enzymatique, gardent les structures et contrôlent les fonctions de parois cellulaires. Il a été constaté que le manque de cations était responsable de la perte d'activité de certaines lactases (enzymes) de β - galactosidase.

# II. PROBLÉMATIQUE.

Il ressort de l'historique bibliographique la problématique suivante :-

- Le lactosérum rejeté comme déchet constitue une charge polluante considérable. Le lactosérum contenant environ 6% (w/w) de solides représente une demande biochimique en oxygène ($DBO_5$) de l'ordre de 30,000 ppm - 50,000 ppm. Les différents voies de valorisation posent certains problèmes. Par exemple, l'ultrafiltration nous permet de concentrer et séparer des protéines du lactosérum et du perméat; mais la présence de lactose (4 - 5% w/v) dans le perméat et sa forte charge en $DBO_5$ pose un problème de pollution.

- La biovalorisation du lactose par la dégradation anaérobie est une alternative attrayante, due au faible coût d'opération et le recouvrement du méthane, réutilisable comme combustible dans l'usine. Puisque le lactose se dégrade facilement en acides(lactique, propanoique , etc), la concentration élevée du lactose dans le lactosérum résulte souvent en un digesteur acidifié dans lequel les bactéries acétogéniques et méthanogéniques ne peuvent bien fonctionner. L'immobilisation de micro-organismes (bactéries) anaérobies sur les parois des bioréacteurs en utilisant leur capacité à s'attacher sur les parois "biofilm" ou à se regrouper " bioflock" peut occasionner des difficultés pour le transfert du substrat

dans les agrégats microbiens. L'usine de traitement ou d'épuration d'effluent de la compagnie d'Agropur du Notre - Dame du Bon Conseil, Québec, Canada, offre un bel exemple où les agrégats microbiens arrêtent le fonctionnement d'un bioréacteur anaérobique.

- Le lactose représente le constituant principal du lactosérum à valoriser à cause de sa charge polluante élevée. L'utilisation directe du lactose est limitée par son faible pouvoir sucrant et certaines personnes présentent des problèmes d'intolérance au lactose. De plus, le lactose cristallin est peu soluble. L'hydrolyse de lactose en glucose et galactose est une alternative potentielle pour surmonter ces handicaps.

- L'hydrolyse du lactose par les systèmes d'enzymes immobilisées a donné de bons espoirs aux industries laitières, de pouvoir mieux valoriser leurs sous produits; mais malheureusement dans la plupart des installations, la demi-vie des catalyseurs biologiques ne sont que de quelques semaines, voire quelques heures. La recherche d'un modèle de vitesse englobant le plus d'influences possible devient nécessaire. Le comportement de l'activité vis-à-vis certains sels ou cations, observé sur les enzymes solubles peuvent être appliqués aux enzymes ou cellules sur supports, mais les essais en réacteurs sont nécessaires pour confirmer leur stabilité.

# III. OBJECTIF

Le but principal de cette étude était de proposer une voie de biovalorisation de l'effluent de fromagerie en fonction de sa composition saline. Cette recherche permet:

1. d'évaluer des paramètres cinétiques en fonction de la concentration en sels du substrat lors l'hydrolyse du lactose.

2. de faire une étude de l'interaction cation-lactose par spectroscopie.

3. d'étudier la biovalorisation du lactosérum en bioréacteurs, réacteur tubulaire (PFTR) et réacteur à réservoir agité (CSTR).

4. de proposer une usine pilote type de biovalorisation des effluents de fromagerie.

5. de faire une étude d'analyse économique de l'usine pilote.

# IV. MÉTHODOLOGIE.

## 4.1. Méthode d'immobilisation.

La méthode d'immobilisation inventée par Chiang, J.P. et Lantero, O.J. Jr, 1990, publiée dans le bureau des Brevets Européen était modifiée et adaptée à cette recherche. Cette méthode consiste à immobiliser le lactase, *Aspergillus oryzae* avec l'activité initiale de 4.4 unités/mg solide avec lactose ou 4.5 unité/mg solide avec 2-Nitrophenyl-β-D-galactopyranoside(ONPG) à 30 °C et pH 4.5 sur un support enzymatique (granular diatomaceous earth). L'efficacité d'immobilisation a été calculée en utilisant l'expression:

$$\text{Efficacité d'immobilisation, } \alpha = \left[\frac{E_o - E_1}{E_o}\right] * 100$$

où $E_o$ = activité initiale d'enzyme.

$E_1$ = activité de la solution résiduelle d'immobilisation.

Après l'immobilisation un test de stabilité thermique d'enzyme immobilisée et un autre pour la stabilité mécanique d'enzyme immobilisée ont été effectués.

## 4.2. Études cinétiques avec du lactose pur.

### 4.2.1. Sans cations.

#### 4.2.1.1 Effet de la température.

L'influence de la température sur les paramètres cinétiques était effectuée avec 2% (w/v) de lactose tout en faisant varier la température de 30 °C à 60 °C.

#### 4.2.1.2. Effet de la concentration initiale du lactose.

L'influence de la concentration initiale du lactose a été mesurée à une température optimale de 50 °C tout en variant la concentration initiale de 0,5% à 5% (w/v).

### 4.2.2. Étude cinétique en présence des cations.

#### 4.2.2.1. Le cas de cation individuels.

Cette étude a été effectuée sur 2% (w/v) de lactose en présence de 4 cations individuels avec les concentrations suivantes: 0.1M NaCl, 0.1M KCl, 2mM $CaSO_4$, et 2mM $MgCl_2$.

#### 4.2.2.2. Le cas de cations combinés

Cette étude a été effectuée sur 2% (w/v) du lactose en présence des combinaisons de 3 cations: $K^+$, $Na^+$, $Ca^{++}$ selon les gammes suivantes:

| | | |
|---|---|---|
| NaCl | : | 0.05M - 0.1M |
| KCl | : | 0.05M - 0.1M |
| $CaCl_2$ | : | 0.5mM - 2mM |

## 4.3. Étude spectroscopique par FT - IR.

Elle a été effectuée avec le lactose libre et ensuite avec le complexe de cation (Na, K, Ca) - lactose.

## 4.4. Hydrolyse des effluents de fromagerie en bioréacteurs

Les substrats utilisés dans cette étude étaient l'effluent de fromagerie (lactosérum) et l'effluent de l'usine d'épuration d'AGROPUR de Notre - Dame du Bon Conseil.

### 4.4.1. Unité d'ultrafiltration (UF).

L'unité d'ultrafiltration était nécessaire pour séparer l'effluent en concentrés protéines de lactosérum et de perméat . Dans ce système la plupart des sels ou cations étaient enlevés lors de ce procédé.

### 4.4.2. Hydrolyse de l'effluent de fromagerie en bioréacteurs.

Le perméat de lactosérum et celui de l'effluent de l'usine d'épuration ont été pompés dans les bioréacteurs; le réacteur à réservoir agité (CSTR) et le réacteur tubulaire (PFTR). À la sortie de réacteur, les produits sont analysés pour la présence de glucose, galactose, lactose, et les oligosaccharides.

Le degré d'hydrolyse, X (%) était calculé en utilisant l'expression :

$$X = \left[ \frac{C_{glucose} + C_{galactose}}{C_{lactose} + C_{glucose} + C_{galactose}} \right] 100$$

ou C est les concentrations des composés trouvées dans les produits obtenus.

### 4.5. Demande biologique et chimique en oxygène.

La $DBO_5$ et DCO des échantillons et les effluents hydrolysés dans les réacteurs ont été mesurées en utilisant le "Dissolve Oxygen Meter" au Centre de recherche en pâte et papier (CRPP).

# V. RÉSULTATS ET DISCUSSIONS.

## 5.1. Résultats d'immobilisation.

### 5.1.1. Efficacité d'immobilisation.

À partir des résultats d'immobilisation obtenus, l'efficacité d'immobilisation d'*Aspergillus oryzae* sur le support, GDE, était 98.4 % et la vitesse optimum d'agitation du bain d'immobilisation était 150 rpm.

### 5.1.2. Stabilité thermique d'enzyme immobilisée.

L'*Aspergillus oryzae* immobilisée était plus stable à 50 °C

### 5.1.3. Stabilité mécanique d'enzyme immobilisée.

L'*Aspergillus oryzae* immobilisée était mécaniquement stable à une vitesse d'agitation optimum du réacteur réservoir agité (CSTR) de 120 rpm.

## 5.2. Études cinétiques.

L'objectifs des études cinétiques étaient de déterminer les paramètres cinétiques, $V_{max.}$ and $K_{m.\,app.}$ et étudier les effets des cations sur les paramètres cinétiques.

### 5.2.1. Études cinétiques sans cations.

Les effets suivants ont été observés en augmentant la température sur les paramètres cinétiques :

- la vitesse maximum de la réaction, $V_{max.}$ , augmente mais elle était limitée aux températures élevées.

- la degré de transformation maximum augmente. Cependant une chute graduelle était remarquée aux températures plus de 55 °C.

- les valeurs de $K_{m\ app.}$ obtenues sont plus faibles que celles trouvées dans certaines revues scientifiques[5,6] en utilisant l'équation de Michealis - Menten avec une inhibition compétitive de produit.

#### 5.2.1.1 Effets de la concentration initiale du lactose sur les paramètres cinétiques

À partir des paramètres cinétiques obtenus, on remarque que lorsque la concentration du lactose augmente :

- la vitesse maximum de réaction augmente mais elle était limitée aux concentrations élevées.

- la conversion diminue, indiquant que le lactose et le galactose sont impliqués dans la formation des oligosaccharides.

- dans la plupart des milieux de réaction la concentration du galactose était inférieure à celle du glucose .

Bien qu'il ait été démontré que les oligosaccharides sont formés pendant l'hydrolyse du lactose, ils étaient négligés dans les modèles de vitesse de réaction proposées. En effet une étude réalisée par S.T. Yang (1980) avait montré que la

formation des oligosaccharides peut être négligée dans la modélisation de la vitesse de l'hydrolyse surtout quand la concentration initiale du lactose est faible et la température de réaction d'environ 50 °C.

## 5.2.2. Études cinétiques en présence des cations.

L'objectif était de démontrer l'influence des cations sur les paramètres cinétiques et aussi de montrer qu'une déminéralisation au complet de l'effluent de fromagerie n'est plus nécessaire avant l'hydrolyse.

### 5.2.2.1. Effets des cations individuels sur les paramètres cinétiques.

À partir des paramètres cinétiques obtenus, on remarque que les paramètres cinétiques sont influencés par la présence des cations. Les cations bivalents en particulier jouent une rôle significatif sur l'activité enzymatique. Ces cations augmentent la vitesse maximale de réaction, $V_{max.}$ de l'ordre de 20% à 60 %. Les paramètres cinétiques étaient très élevés pour $Ca^{++}$ et $Mg^{++}$ par rapport au $K^{+}$ et $Na^{+}$ ou en absence de cations. On a noté aussi que les paramètres cinétiques pour les cations monovalents étaient moins élevés que ceux en absence de cations. Les taux de transformation étaient plus élevés chez les cations bivalents que les monovalents.

#### 5.2.2.2. L'influence de cations combinés.

Les résultats obtenus en variant les concentrations des cations combinés étaient compliqués et parfois difficiles à interpréter parce que les paramètres cinétiques n'étaient pas uniformes. Cependant, la vitesse maximum de réaction la plus élevée était 6.63 $mmol.L^{-1}.min^{-1}.g^{-1}_{IME}$ et la valeur $K_{m.app.}$ était 72.69 mmol./L. Ces paramètres cinétiques ont été observés à une concentration optimum de 3 cations, Na : K : Ca dans le rapport 75mM : 75mM : 0. 5mM respectivement. Les valeurs de $K_{m.app.}$ obtenues en présence des cations étaient faibles, indiquant ainsi, une grande affinité d'enzyme pour lactose. Par conséquent, l'attachement de l'enzyme au complexe de cation - lactose est très forte.

## 5.3. Études spectroscopique avec le FT-IR.

L'étude spectroscopie était l'une de nouveauté de ce projet de recherche. Aucune étude sur l'interaction de lactose - cations n'a été réalisé jusqu'à présent. Les résultats des spectres obtenus dans la marge de 1800-1000 $cm^{-1}$ sont analysés dans cette étude. Un seul groupe de bande d'absorption a été observé à 1650 $cm^{-1}$ . La bande d'absorption a montré un changement d'intensité considérable en complexant le lactose avec les cations. La variation de l'intensité observée avec une concentration de 0.5M de cation monovalent ($Na^+$ et $K^+$ ) a montré que l'absorption était plus forte pour $Na^+$ que pour le $K^+$ . Cette dissimilarité est due au rayon ionique de $Na^+$ qui est petit par rapport à celui de $K^+$.

Pour le complexe de $Ca^{++}$ - lactose, on a observé une absorption remarquable et distincte à 1650 $cm^{-1}$ , mais aux concentrations de 0.5 - 1M $CaCl_2$ la bande d'absorption est déplacée à une fréquence inférieure de 1637 $cm^{-1}$.

L'ordre d'intensité d'absorption est Ca > Na > K. Ceci montre que l'interaction entre le calcium et lactose est plus forte que celles observées entre $Na^+$ ou $K^+$, indiquant que le complexe cations bivalents ($Ca^{2+}$, $Mg^{2+}$ ) a beaucoup d'affinité pour l'enzyme que les cations monovalents ($Na^+$, $K^+$ ). Ceci explique la valeur élevée de vitesse maximum, $V_{max.}$ , et un degré élevé de l'hydrolyse par rapport aux cations monovalents. Cela peut être confirmé par le fait que les valeurs de la constante de Michealis - Menten, $K_{m.app.}$ obtenues dans cette étude était plus faible pour les cations bivalents que les cations monovalents. Plus que $K_{m.app.}$ est petit, plus l'affinité d'enzyme pour le substrat est forte.

## 5.4. Hydrolyse de l'effluent fromagerie dans les bioréacteurs .

Le degré de l'hydrolyse était 30% plus élevé dans réacteur tubulaire (PFTR) que le réacteur réservoir agité (CSTR). Pour atteindre le même dégrée de conversion, le CSTR demandera plus d'enzymes immobilisées que le PFTR. La demi-vie d'enzyme immobilisé était 17 jours. Friends and Shahani [7] en utilisant le même enzyme et les mêmes conditions opérationnelles à une température, 50 °C ont trouvé une demi-vie de 13 jours. Le désactivation d'enzymes était influencée par

la température d'opération, le pH, la nature de substrat et la contamination microbienne.

## 5.5. Étude d'analyse économique.

L'objectif de l'étude économique était de démontrer la faisabilité industrielle de ce projet et de déterminer si l'usine sera installée en parallèle ou en série avec l'usine d'épuration existant à l'usine de fromage d'AGROPUR de Notre -Dame du Bon Conseil, Québec, Canada.

L'usine pilote devra traiter 48 $m^3$ or 50 tonnes par jour. Les prix des équipements étaient obtenus soit chez les manufacturiers, soit chez les distributeurs soit à partir des graphiques et mis à jour en utilisant le "Chemical Engineering plant cost Index " pour faire le rapport des coûts pour l'année 1995.

Le fonds de roulement est le capital nécessaire pour le fonctionnement du procédé. Le fonds de roulement varie entre 10 - 20 % de capital fixe dépendant de la complexité de l'usine et du coût de matière première. Le capital total qui est la somme total à investir par les investisseurs a été estimé à 3,695,538 $. Le coût total du produit comprend les coûts de fabrication et les dépenses générales. Le coût total du produit dans cette étude était estimé à 1.4 millions $. Les revenus étaient de 1.6 millions $. Ce qui a donné un profit net après impôt de 100,000$

# VI. CONCLUSION.

Cette recherche a bien démontré que les cations jouent une rôle significatif sur l'activité et la stabilité du lactase, *Aspergillus oryzae*. Une analyse de l'effet des spectres de FT -IR des cations sur la cinétique de l'hydrolyse du lactose a confirmé qu'ils réagissent en augmentant l'affinité du complexe cation - lactose de l'enzyme et l'activité maximum de l'enzyme.

Le $Ca^{++}$ et $Mg^{++}$ étaient activateurs, alors que les cations monovalents ( $K^{+}$, $Na^{+}$ ) étaient stabilisateurs pour la lactase, *Aspergillus oryzae*. Cependant, ces cations peuvent devenir inhibiteurs lorsque leurs concentrations dépassent certaines limites. Par conséquent, il est très important de contrôler les concentrations cationiques dans le milieu réactionnel.

À partir des caractéristiques spectroscopiques du lactose étudiés ici, on peut tirer les conclusions suivantes :

- La forte liaison d'hydrogène du lactose libre est maintenue après le complexation;
- Il y a quelques interactions entre cations et les groupes OH, aussi que C - O du lactose anion.

Le réacteur tubulaire est plus performant, plus efficace, et plus économique que le réacteur en agitation continu pour l'hydrolyse du lactosérum.

Le $DBO_5$ était réduit de 33,3000 mg/L pour l'effluent fromagerie (lactosérum) à 300 mg/L pour l'effluent hydrolysé tandis que l'effluent de l'usine d'épuration existante était réduit de 2,300 ppm à 84 ppm donc un niveau plus bas que la limite de 1,000 mg/L stipulée par le Ministère de l'Environnement pour les industries laitières.

À partir d'étude d'analyse économique on peut conclure que l'usine pilote sera rentable si elle est installée en parallèle à l'usine d'épuration existante dans la fromagerie de Notre-Dame du Bon Conseil.

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UNVERSITÉ DU QUÉBEC

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN ENVIRONMENTAL SCIENCES

BY

NYEANGO JOHN NJIKE

# THE VALORIZATION AND THE INFLUENCE OF WHEY CATIONS ON THE BIOTRANSFORMATION OF CHEESE INDUSTRIAL EFFLUENT.

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# RÉSUMÉ.

La valorisation de l'effluent de fromagerie par l'hydrolyse avec les enzymes immobilisées sur supports en sirop de glucose et galactose constitue une double solution pour la gestion des effluents de fromagerie. Elle permet de satisfaire les attentes économiques de l'industrie fromagère et de répondre aux exigences environnementales de la société. Dans cette optique, nous avons conçu deux types de réacteurs, un réacteur tubulaire (PFR) et un réacteur en agitation continue (CSTR). Chacun de ces réacteurs était rempli d'une quantité d'enzyme immobilisée sur diatomée (GDE) en fonction du volume réactionnel.

Des essais préliminaires de l'hydrolyse de lactose pur par différentes B-galactosidase ont permis de retenir l'enzyme *Aspergillus oryzae*. Des essais d'optimisation des conditions d'immobilisation d'enzyme immobilisée ont conduit au choix d' une vitesse d'agitation optimum de bain d'immobilisation de 150 rpm qui a donnée une efficacité d'immobilisation plus de 98% et une stabilité thermique de 50 °C. Des tests d'optimisation des conditions d'opération d'enzyme immobilisée ont conduit au choix d'une stabilité mécanique à 120 rpm pour le CSTR, un débit de 40 ml/min pour le PFR, et un pH 4,5 pour le milieu réactionnel. L'hydrolyse de lactose en présence et en absence de cations a démontré que les cations bivalents ($Mg^{2+}$, $Ca^{2+}$) influencent beaucoup la vitesse de réaction de l'hydrolyse et par la suite augmentent l'activité d'*Aspergillus oryzae*.

Les expériences dans les bioréacteurs ont prouvé que le réacteur tubulaire est plus efficace que le CSTR pour l'hydrolyse du lactose et ont donné un rendement de conversion de plus de 85 % pour les effluents fromagerie.

De plus une étude économique de l'usine pilote proposée à partir de ce projet recherche permet d'avancer que l'application industrielle de ce type de procédé pourrait être rentable.

Finalement une étude spectroscopie par FT-IR, nous a permis de faire une tentative pour expliquer le mécanisme de l'interaction cation - lactose qui n'a jamais été réalisé, cependant d'autres études seraient nécessaires pour valider cette théorie avancée. Les cations bivalents ont augmenté la vitesse de réaction maximum, $V_{max.}$ de l'hydrolyse du lactose par 4 à 60%.

# ABSTRACT.

Lactose in reconstituted whey permeate was hydrolyzed using immobilized β-galactosidase from *Aspergillus oryzae*. The effect of single as well as a combination of three whey cations (Na, K, and Ca) at different concentrations on the hydrolysis of lactose were studied. Divalent cations significantly increased the rate of lactose hydrolysis. The maximum rate of reaction, $V_{max}$, was increased by 60% in the presence of $Mg^{2+}$. A FT-IR spectroscopic study also confirmed the important role played by divalent cations on the kinetics of lactose hydrolysis. When a combination of these three cations were utilized, a maximum rate of hydrolysis, $V_{max}$, of 6.63 mmol/L.min.$g_{IME}$ and $K_{m.app.}$ of 72.69 mmol/L. was obtained at a concentration of 75 mM, 75 mM and 0.5 mM for Na, K, and Ca respectively. High conversion (85%) of lactose was achieved in less than 1 hr. residence time at 50 °C and pH 4.5 in a plug flow tubular reactor. The biological oxygen demand($BOD_5$) of the substrate was reduced from 35,000 ppm to about 300 ppm.

**This thesis is dedicated to my parents Mr. and Mrs. Nyeango, and to my son Nyeango Jimmy Liu whose support contributed to the completion of this work.**

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# 1. INTRODUCTION AND PROBLEM DEFINITION.

## 1.1. INTRODUCTION

The production of cheese in Québec and Canada has been increasing steadily and according to Statistic Canada (1) it attained 159.5 * $10^3$ tons and 307.2 * $10^3$ tons respectively in 1994. Cheese manufacturing is accompanied by the production of large quantities of high strength liquid effluent (whey) that constitutes a major disposal problem. It is estimated that 2.203 million tons of cheese whey was produced in Canada in 1994.

Historically, cheese industrial effluent was considered as waste, and was either given to animals as feed, spread on land, farms, or discharged into rivers and municipals sewage systems. Due to stringent environmental regulations, coupled with the high cost of using municipal treatment systems; cheese manufacturers have increasingly been viewing cheese industrial effluent as a rich and cheap source of raw material from which valuable by-products could be obtained. Cheese industrial effluent contains lactose, protein, mineral salts, fats, lactic acid and water, [Table 1]. Whey is a very rich source of mineral salts such as Calcium, Copper, Iron, Phosphorus, Potassium and Sodium. It is also a potential source of vitamin of the B-complex, riboflavin and other vitamins. Whey contain a 6% (w/v) total solid of which the principal constituent is

lactose(70% (w/w)). In 1990, Keller[2] proposed that whey could be disposed according to four "Fs" in order of hierarchy ;

Food : human consumption as whey protein (WPC), glucose, or vitamin.

Feed : for animal (pigs and chicken) consumption.

Fuel : fermented to methane or ethanol

Fertilizer: land spreading because it contains nitrogen, phosphorus and potassium

The challenge of finding a solution to the problems caused by cheese industrial effluent has attracted the attention of researchers, government and the dairy industry because of nutritional (lactose intolerance), technological (crystallisation), and environmental (pollution) problems associated with it [3,4,5]. For the past two decades, most researchers have put greater emphasis on the hydrolysis of lactose by the enzyme β - galactosidase. The immobilization of this enzymes offers the advantage of repetitive use and application in continuous systems thereby reducing the processing cost as well as eliminating enzyme contamination problem. Although a few researchers have investigated and shown that metal ions or cations play a crucial role on the activity and the stability of some of these enzymes during the hydrolysis of lactose [6,7,8], published information on the modification of lactose hydrolysis kinetic parameters caused by whey cations are limited. Moreover, no phenomenological explanations of these modifications or the interactions of cations - enzyme-substrate complex has been reported in literature.

Table 1

Composition of whey and whey permeate [9,85]

| Components (%w/w) | Sweet whey | Acid whey | Whey permeate[85] |
|---|---|---|---|
| Total Solids | 6.67 | 7.37 | 6.81 |
| Moisture | 93.33 | 92.63 | 93.19 |
| Lactose | 5.14 | 5.12 | 5.10 |
| Protein | 0.76 | 0.76 | 0.30 |
| Fats | 0.02 | 0.09 | 0.01 |
| Ash(mineral salts) | 0.60 | 0.80 | 0.80 |
| Lactic acid | 0.15 | 0.60 | 0.60 |

## 1.2. PROBLEM DEFINITION.

A thorough review of the dairy industry especially cheese manufacturing factories has led to the following problems concerning its effluent :

- Cheese industrial effluent discarded as waste creates severe pollution problem. Cheese industrial effluent and its permeate each with a total solid content of about 6% (w/v) represents a biochemical oxygen demand ($BOD_5$) of 30.000 ppm to 50.000 ppm [9,10] . The different methods of valorization present certain problems; for example ultrafiltration which has emerged as one of the best ways of valorizing cheese industrial effluent to obtained a protein-rich ingredient generates large quantities of permeate containing 4% to 5% (w/v) of lactose with a BOD of more than 30,000 ppm. This indicates that ultrafiltration is not the best.

- The biovalorization of lactose by anaerobic degradation is an attractive alternative due to the cheap operational cost as well as the recovery of methane gas which can be reutilized in the cheese factory as heating fuel. However, lactose is decomposed easily into acid, and the high concentration of lactose in cheese industrial effluent often leads to a sour digester in which the acetogenic and methanogenic bacteria can't operate properly [11, 12,13 ] .

The immobilization of anaerobic bacteria on the wall of the bioreactor or fermentor as well as the regrouping of these microbes together (bioflock) can lead to difficulties of the substrate transfer in the microbial aggregate.

- Lactose is the main constituent in cheese industrial effluent to be valorized due to its high pollution strength. Direct utilization of lactose is limited due to its low sweetness and the problem of lactose intolerance shown by certain persons, in addition to the low solubility of lactose[14]. The hydrolysis of lactose to glucose and galactose is a potential alternative to overcome these handicaps.

- The hydrolysis of lactose by immobilized enzymes have given good hopes to dairy industries, to better valorize their by-products, but unfortunately in most of the installations, the half - life of the biocatalyst is just a few weeks, some even a few hours. The need to test a reaction model that will englobe most of the influencing factors becomes a necessity. The effect of enzyme activity with respect to certain salts or cations, observed in soluble enzymes could be applied to enzymes or cells on supports or carriers. However, assays in bioreactors are necessary to confirm their stability. Finally, there is the economics problem. Immobilized enzymes can be recycled and reused leading to low operational cost for immobilized systems as compared to using soluble enzymes.

## 1.3. OBJECTIVES

The principal objective of this research is to propose a method for the biotransformation of cheese industrial effluent as a function of its saline (cation) composition. This study would enable us to :

1. Evaluate the kinetic parameters as a function of the concentration of metal ions present in cheese factory effluent.

2. Investigate the biovalorization of cheese factory effluent in bioreactors; the plug flow reactor and the continuous stirred tank reactor (CSTR).

3. Propose a pilot plant for the biotransformation of cheese industrial effluent and the separation of the resulting by - products, the whey protein concentrates (WPC35%) and the hydrolyzed syrup .

4. Make a technico - economic evaluation (total capital estimation, product estimation and profitability) of the pilot plant.

5. Propose a tentative explanation for the mechanism of cation - lactose complex interaction.

# II. LITERATURE REVIEW

## 2.1. Definition of cheese industrial effluent.

Cheese industrial includes whey, waste water, detergents and disinfectants used for cleaning and disinfecting cheese manufacturing equipment. Whey is a greenish - yellow fluid obtained by separating fats and casein from whole or skim milk. Whole milk is used for the production of natural cheese such as cheddar and the resulting by-product is known as sweet whey. It has a pH greater than 5.0. Sweet whey is generated at a proportion of nine (9) kg for every kg of cheese manufactured. Acid whey is derived from the manufactured of cottage cheese, lactic casein or mineral acid casein from skim milk. It has a pH less than 5.0. Acid whey is generated at the rate of six (6) kg for every kg of cottage cheese produced. This low pH value is due to the acid produced mainly lactic acid and / or used for coagulation.

In Québec the normal process of cheese manufacturing leads to a spillage loss which represents between 1 and 12 % of the milk received. The average is situated at about 5% [15] . This variation depends on three important factors:

- the age of the cheese factory;
- the way the piping system was installed;
- the environmental conscience of the factory.

Annex 1 presents a flow sheet of the liquid waste generated in a cheese factory.

Waste water including chemicals (detergent and disinfectants) used for cleaning and disinfecting equipment in dairy industries constitutes an important source of waste generation in cheese industries. Other pollutants generated from the cheese factory include lubricants from salts of fatty acids.

Lactose is the main constituent of cheese factory effluent. Discharging this effluent in water or spreading on agricultural farmland without appropriate pre-treatment would favour the development a filamentous bacterial population which are incapable of utilizing the amino acids present as a source of nitrogen. The large size of the protein molecules are unable to pass through the bacterial cell wall, consequently the excess of nitrogen can increased the level or concentration of nitrate in water thereby modifying its pH. The amount of whey that can be spread on land as fertilizer is limited to 45 - 90 tonnes per acre [16]. Exceeding these limits poses a threat to environmental pollution from run - off and off - odour production. The discharge of cheese industrial effluent can cause special problems in relation to treatment plants due to variation in volume and strength, temperature, pH value, nutrient levels, fat and curds levels[17] .

## 2.2. Methods of valorizing cheese industrial effluent.

The valorization of whey can be classified into two processes namely :

Physico- chemical process and the biotechnological process.

### 2.2.1 Physico-chemical process

This include ultrafiltration, reverse osmosis, evaporation and drying, ion exchange or electrodialysis. These techniques permit the separation, modification, and recombination of whey constituents to obtain several products used in numerous sectors of the food industries. Evaporation and drying is the most widely used method to produce whey powder which is used in bakery, other food preparation and animal feed. This process is economical for large cheese plants with output of more than 150.000 litres of whey per day, but it is unprofitable for small and medium size cheese plants. About 60 % of whey generated in Québec is dried or given to hogs as feed. Statistics show that about 70 % of Québec's cheese manufacturing plants produce less than 50,000 litres of whey per day, representing thus, the principal source of whey in Québec. Scattered in far location and small quantities of whey produced, accompanied by inadequate storage conditions are the main obstacles facing small size cheese

plants with output of less than 50,000 L of whey per day. However, in small size cheese plants whey may be transformed to fermented ammoniated concentrated whey (FACW) used as animal feed or spread as fertilizers on farms [9].

Ultrafiltration offers an alternative way of treating cheese industrial effluent by separating it into a much more valuable whey protein concentrate (WPC) and a lactose stream. The WPC has a significant sale value and the North American market for WPC (35 % - 75 %) attained about 150 million U.S. dollars in 1995 [5]. The 35 % WPC is as a substitute for skim milk powder and the 75 % WPC can replace egg white in baking[18]. The main disadvantage of UF is the high water content (about 93%) in the UF permeate , coupled with the presence of lactose (4 - 5 %) and posing a disposal problem, besides being a perishable product.

Reverse Osmosis permits the concentration of of lactose in the permeate but its high energy consumption makes this process costly. Modler [19] suggested that reverse osmosis should be used in medium size cheese plants with a daily output of about 50.000 to 150.000 L of whey.

### 2.2.2 Biotechnological process.

This process enable the transformation of lactose into a variety of products. It includes hydrolysis and fermentation. Their products are designated mostly to non-food markets namely; pharmaceuticals, chemicals, or cosmetics.

The hydrolysis of lactose generates in theory an isomolecular mixture of two (2) sugars; glucose and galactose. But , in reality oligosaccharides are also generated by the polymerization of galactose or a reaction between galactose and lactose [20,21] . These oligosaccharides are undesirable products. The quantity of oligosaccharide produced can be minimized by : using a fungal enzyme; keeping initial lactose concentration to a minimum; using immobilized enzymes because of high mass transfer effect.

The hydrolyzed lactose enable us to obtain by-products with increased solubility and sweetness, as well as producing syrups which are microbiological stable, thereby avoiding the process of drying. These products can be consumed by people suffering from lactose intolerance. Lactose intolerance or malabsorption is a problem that arises when there is insufficient enzyme in the intestine to hydrolyzed all the lactose ingested. Consequently, the unhydrolyzed lactose is not absorbed and move to the large intestine where bacterial actions and osmotic

effects often cause abdominal discomfort, bloating and flatulence. Lactose can be hydrolyzed by two methods : Acid hydrolysis and enzymatic hydrolysis.

Acid hydrolysis can be carried out by either a homogenous reaction in which the acid is free in solution or by a heterogenous reaction catalyzed by hydrogen ions. This method is simple and gives a high degree of conversion in a shorter time compared to enzymatic hydrolysis of lactose. However, it can't be applied to milk hydrolysis and protein containing lactose solution (cheese factory effluent) because of protein denaturation and coagulation at high temperature and low pH [22,23].

Enzymatic hydrolysis of lactose is carried out by soluble (free) enzymes or enzymes immobilised on biocatalyst carriers. The method by enzymes immobilized on carriers is preferred because :

- immobilized enzyme can be recycled and reused, thus improving enzyme utilization and the overall productivity;
- immobilized enzymes can be used in a continuous system;
- immobilized enzymes system give products of high purity and yield;
- low cost of operation for immobilized enzymes systems;

- immobilized enzymes are more resistant to thermal deactivation than soluble enzymes.

The rate model of reaction for lactose hydrolysis into glucose and galactose is often represented by the apparent constant of Michealis-Menten, $K_{m.app.}$ and the apparent maximum rate of reaction, $V_{max.app.}$. The numerous enzymes - carrier systems studied have been characterized by parameters such as pH, temperature, substrate concentration, enzyme concentration, residence time, stirring and mixing conditions, thereby defining the optimal operating conditions.

The utilization of whole cells can avoid the costly and crucial process of extraction and purification of enzymes before immobilization on supports [24]. Despite the enormous quantity of β - galactosidase - carrier systems developed, very few have been exploited in pilot plants or at industrial level. Most probably because of the following reasons :

- problems of microbial contamination in continuous systems;

- losses in enzyme activity as result of immobilization;

- immobilized enzymes could suffer from steric hindrance effects;

- thermal degradation of enzymes as well as enzyme inhibition by hydrolyzed products; galactose, oligosaccharides, or accumulated substrate [20] .

- harmful effects of salts or cations on the enzyme system.

A preliminary step of partial demineralization of the substrate by ultrafiltration or by membrane process can resolved the harmful effect of cations on the enzyme system.

## 2.3. The Case of β - galactosidase and the influence of cations.

β - galactosidase is the trivial name for the enzyme lactase ( E.C.3.2.1.23). Typical β - galactosidase has a multi - subunit structure. Metal ions play a crucial role on the activity and stability of a typical β-galactosidase. Some lactases require metal ions for their activation, others for their stability and some for both activation and stability. Table 2 summarizes some lactase enzymes and the type of cations that influences their activity and / or stability. The β-galactosidase from *E. coli* and *K. lactis* are activated by $Na^+$ and $K^+$ but don't need monovalent or divalent cations for their stability. The β-galactosidase from Kluyveromyces fragilis specifically requires $K^+$ for its stability . West et al., [25] investigated β-galactosidase from yeast, *Trichosporon cutaneum* and concluded

**GENERAL PROPERTIES OF SOME COMMERCIAL LACTASES[33,35,36]**

Table 2.

| | Fungal lactase | | Yeast Lactase | | Bacteria |
|---|---|---|---|---|---|
| Source | A. niger | A. oryzae | Lactozyme | S . lactis | E. coli |
| Opt. pH | 3.0-4.0 | 4.5 | 6.5 | 6.8 - 7.0 | 7.2 |
| pH range | 2.5- 4.5 | 3.5 - 5.5 | 40 | 6.0 - 8.5 | 6.0 - 8.0 |
| Opt. temp. | 55 | 50 | 37 | 35 | 40 |
| Temp. range | 30 - 60 | 30 - 55 | 30 - 45 | 20 - 55 | 30 - 65 |
| Activators | NIL | NIL | - | $Mg^{++}$ | $K^{+}$, $Na^{+}$ |
| Stability | NIL | NIL | - | $Mn^{++}$ | $Mn^{++}$ |
| Producers[a] or Distributors[b] | SIGMA[b] | SIGMA[b] | NOVO-Lab.[a] | GIST-BROCADES[a] | SIGMA[b] |

that, this enzyme needed $K^+$ and $Rb^+$ for its activity and stability. However, divalent cations like $Mg^{++}$ and $Mn^{++}$ stimulated its activity to a lesser extend. A study by Chang et al., [26] on factors affecting the thermostability of β - galactosidase from streptococcus salivarius in milk showed that $K^+$, $Mg^{++}$ and $Ca^{++}$ had a major contribution on its stability. It was observed that the absence of $Mg^{++}$ or $Ca^{++}$ in the saline composition of milk led to a drop in stability by 62 % and 50 % respectively, while the complete absence of these two cations resulted in a drop in the stability by 85 %.

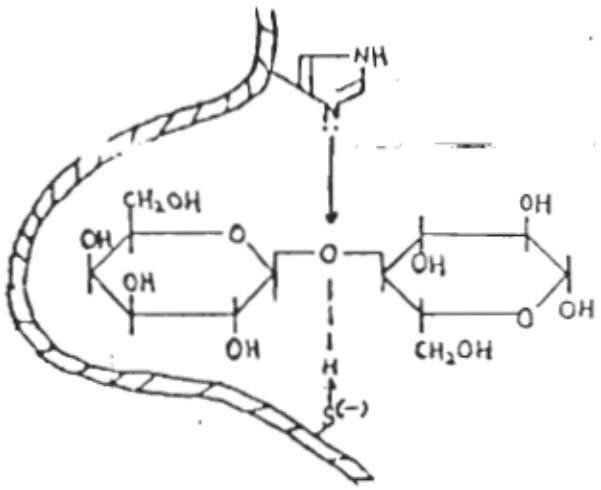
One of the areas that remains unexploited is the variation of the rate model of hydrolysis as a function of cheese whey cations. The analysis of whey by B. Menten et al., [27] showed the presence of cations such as $Ca^{++}$, $Mg^{++}$, $K^+$, and $Na^+$. The choice of an appropriate β-galactosidase that would require these cations for their activation and stability could give different results for $V_{max}$ and $K_m$ or simply modify the rate of hydrolysis.

## 2.4. Action of β - galactosidase.

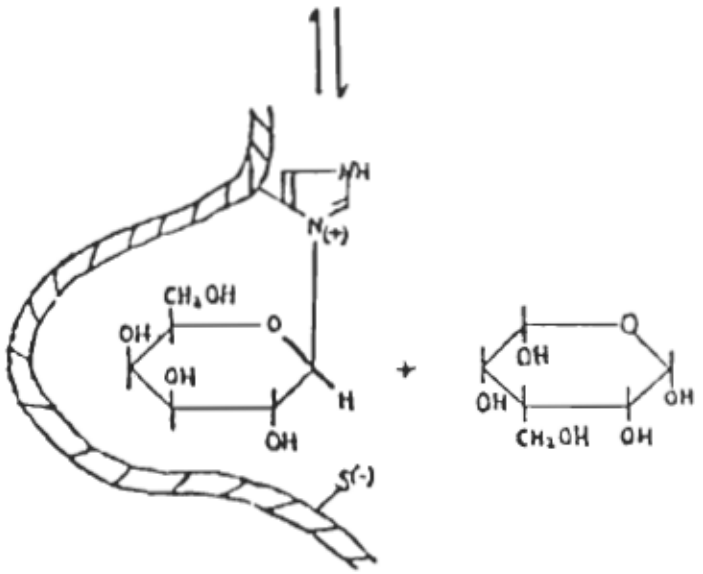
The enzyme, β - galactosidase typically catalyzes the hydrolysis of β-D-galactosides (lactose, ONPG) and α-L-arabinoside [28]. The active site of β -

galactosidase for this hydrolysis reaction is the imidazole and the sulfhydryl groups[29] . The imidazole group attacks the nucleophillic centre at the first carbon of the galactose molecule while the sulfhydryl group protonates the galactosidic oxygen atom as depicted in figure 1. β - galactosidase attacks the O - galactosyl group of lactose during hydrolysis. According to Wallenfels et al.[30], only the D - pyranoside ring will be hydrolyzed, leading to the formation of the enzyme - galactosyl complex with the simultaneous liberation of glucose, leaving the β - glucosidic bond intact.

β - galactosidase is capable of catalyzing hydrolytic reactions as well as transfer reactions. J.E.Presnosil et al. [20], explained that the enzyme transfers the galactose moeity of β - galactosidase to an acceptor containing a hydroxyl group as shown in figure 2. If the acceptor is water, galactose is formed and liberated at the active site. In this case the lactase should be considered a transferase rather than a hydrolase [30] . However, if other sugar present in the solution ( e.g., lactose ) serve as an acceptor, a new sugar, oligosaccharide would be formed. This β - D-galactosyl transfer occurs preferentially at the primary alcohol site of D - glucose with the formation of various di - and oligosaccharides [31].

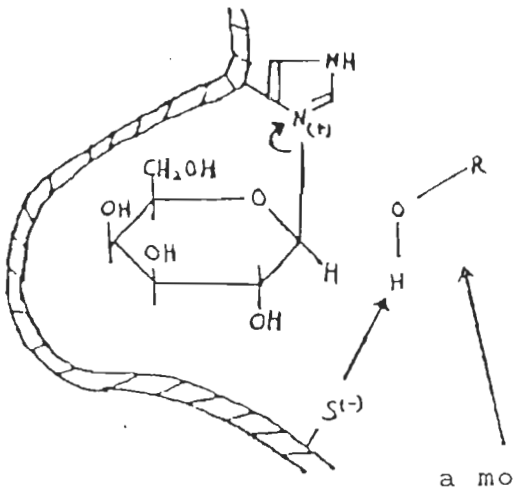



A 4-O-β-galactopyranosyl-D-glucopyranose molecule at the active site of β-galactosidase enzyme

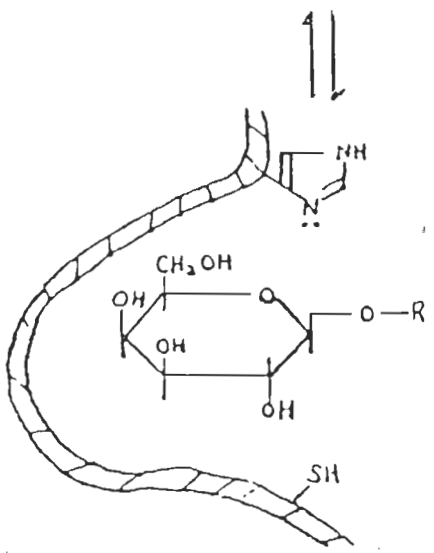



β-Galactosidase-galactose complex + glucose

Figure 1. Proposed Mechanism of Lactose Hydrolysis By β-Galactosidase.




β-galactosidase-galactose complex




a di- or oligosaccharide prior to release from the active site of β- galactosidase

Figure 2. Proposed Mechanism of Galactosyl Transfer Reaction by β-Galactosidase.

## 2.5. Properties and application of β - galactosidase.

The properties of each enzyme depends on the source of the enzyme. The catalytic activity of an enzyme is affected by pH, temperature, ionic strength, inhibitors and substrate concentration [32] . The immobilization of enzyme and the type of carrier used influences the optimal pH and temperature of the enzyme. Properties, cost, availability and safety of β - galactosidase are important factors to be consider for any full - scale enzymatic process for lactose hydrolysis. Fungal enzymes, *Aspergillus niger* and *Aspergillus oryzae* with optimum pH 4.0 - 5.0 are ideally suitable for acid whey (pH 4 - 5) hydrolysis while yeast; *Kluveromyces fragilis, Sacchromyces lactis* with optimum pH 6.5 - 7.0 and bacterial (*E.coli*, pH 6.5 - 7.5) lactases are suitable for milk ( pH 6.6) and sweet whey (pH 6.1) . Table 2 gives general characteristics of some commercial lactases. Fungal lactase is more thermostable than yeast and bacterial lactases, retaining their activities at a temperature of 55 °C for prolonged periods. This gives them two main advantages : higher conversion or shorter residence time for a given conversion rate compared to other lactases and less prone to microbial contamination.

β - glactosidase preparations from fungi ( *A. niger* & *A. oryzae*) and from Yeast ( *K. fragilis* and *S. lactis* ) are generally recognized as safe (GRAS)

because these sources have a long history of safe used in food processing and have been subjected to numerous test [33]. Lactase (*E.Coli*) from bacteria is not considered safe in food processing because it gives toxicity problems with crude extracts of coliforms [34] and it is expensive. Based on these properties, β - galactosidase from *Aspergillus oryzae* was chosen for the hydrolysis of cheese industrial effluent with pH 4.9, used in this study.

## 2.6. IMMOBILIZATION OF β - GALACTOSIDASE

### 2.6.1. Method of immobilization.

Immobilized enzymes are enzymes that are physically confined or localized in a microenvironment with retention of their catalytic activities and can be used repeatedly and continuously. The art of enzyme immobilization is a rapidly expanding and advancing field. During the past two decades numerous techniques have been developed and used for the immobilization of β - galactosidase in lactose hydrolysis. These techniques can be classified as : Intramolecular Cross - Linking, Covalent Binding, physical Adsorption, Ionic Binding, Metal Binding, and Entrapment.

The first two are generally classified as chemical method because they form covalent bonds between the enzyme and the carrier (support), while the other are physical methods. A comparative characteristics of the different immobilization technique is given in table 3.

### 2.6.2. Enzyme carriers or supports.

Many enzyme carriers have been successfully developed and used to immobilized β-galactosidase [33,38,39]. Selecting a carrier involves defining the properties which are most important in the carrier. It is important to consider the following factors before choosing an enzyme support :

- suitable particle size and pore diameter to maintain excellent diffusion properties in reactors and high production rate.

- suitable pore size to provide large surface area and to avoid steric hindrance for both enzyme and substrate.

- structural stability over a wide range of temperature, pH, pressure and solvent composition.

- posses functional groups which is required for covalently bound immobilized enzymes and can be activated or modified without harming the carrier structure.

**Table 3:**

**Comparison of attributes of different immobilization techniques**[32, 37]

| Properties | Cross - linking | Covalent binding | Physical Adsorption | Ionic binding | Metal binding | Entrapment |
|---|---|---|---|---|---|---|
| Preparation | Medium | Difficult | Simple | Simple | Simple | Difficult |
| Binding force | Strong | Strong | Weak | Medium | Medium | Medium |
| Enzyme activity | Low | High | Medium | High | High | Low |
| Stability | High | High | Low | Medium | Medium | High |
| Carrier regeneration | Impossible | High | Possible | Possible | Medium | Medium |
| Immobiliza - tion Cost | Medium | High | Low | Low | Medium | Medium |

- physical durability for long- run operations and chemical inertness to microbial attack.
- good regeneration capacity and low cost
- insoluble supports can be used in several industries; for example: food and dairy industries.

Based on these criteria, silica and silicates base materials; celite, granular diatomaceous earth(GDE), glass, and ceramics appears to be better than others for the hydrolysis of cheese effluent. They are compatible with a very wide range of aqueous and organic environments. They are presently being used for pharmaceuticals, beverages, petrochemicals, wastewater treatment systems and drug filtration. It can be concluded from literature [40, 41] that diatomite(GDE and celite) are the most suitable carriers for the preparation of adsorption type immobilized enzymes.

## 2.7. Enzyme reactor.

Different types of reactors have been used with enzymes in their free or immobilized forms. These reactors can be classified according to their mode of operation and

reactor flow patterns. The flow patterns ranges from the well-mixed continuous - flow stirred tank reactor (CSTR) to the " ideal " plug flow (PFR) or tubular reactor.

The plug flow is kinetically more efficient than the continuous stirred tank reactor systems on the basis of product formed per unit time per unit enzyme activity. The well - mixed continuous stirred tank reactors have the following characteristics [42] : Low cost and relatively simple, flexibility in use and stability for many processes, excellent mixing and control conditions as well as low reactivity per unit volume. Recycled loops may be used with the stirred tanks to allowed separation of the immobilized enzyme from the product stream. This necessitates either the retention of the enzyme in the reactor or its recovery from the product stream. The choice of a reactor system must take into consideration the following factors [37] :

- reactor utilization and cost: continuous operation offer several advantages, for example diminished labour costs, the facilitation of automatic control, greater constancy in reaction conditions, a more reliable product and enzyme reuse.

- operational requirements : easily control in a well-mixed system, fresh immobilized enzyme can be added without interrupting the process for a CSTR but for many other systems the reactor must be shut down.

## 2.8. THEORY

### 2.8.1 Kinetics of lactose hydrolysis by β-galactosidase

Theoretically, the enzyme β-galactosidase catalyzes lactose hydrolysis reaction to liberate an isomolecular mixture of glucose and galactose.

$$\text{lactose} \xrightarrow{\beta - \text{galactosidase}} \text{glucose} + \text{galactose}$$

The Michealis-Menten rate model for lactose hydrolysis represented as :

$$v = -\frac{ds}{dt} = \frac{dp}{dt} = \frac{V_{max}[S]}{K_M + [S]} \qquad (1)$$

where v represents the rate of substrate disappearance or the rate of glucose formation, and S represents the substrate concentration.

Several researchers [43, 44, 45, 46] have investigated the kinetics of lactose hydrolysis with fungal enzymes and the Michealis - Menten kinetics with competitive product inhibition by galactose has been widely used as the kinetic model for this reaction.

The molecular event involved in this mechanism can be depicted by :

$$S + E \rightleftharpoons ES \longrightarrow E + P + Q \qquad (2)$$

$$E + P \rightleftharpoons EP \qquad (3)$$

$$v = -\frac{ds}{dt} = \frac{k_3 E_o S}{\left[K_m\left(1+\frac{P}{K_i}\right)+S\right]} \qquad (4)$$

where ES = enzyme - substrate(lactose) E = free enzyme

EP = enzyme-product(galactose) P = galactose

Q = glucose. $k_1, k_2, k_3, k_4, k_5$ = primary reaction rate constants.

t = reaction time. $V_{max.} = k_3E_o$ = maximum reaction rate.

$K_m = (k_2 + k_3)/k_1$ = Michealis constant. $K_i = k_4/k_5$ = inhibition constant.

However, recently S.T. Yang (1989)[47] showed that the reaction mechanism for the hydrolysis of lactose could be described by the following equations:

$$S + E \rightleftharpoons ES \longrightarrow (EP) + Q \qquad (5)$$

$$(EP) \rightleftharpoons E + P \qquad (6)$$

$$v = -\frac{ds}{dt} = \frac{k_3 EoS}{K_m\left(1+\frac{P}{K_i}\right)+(1+K_1)S} \qquad (7)$$

where $K_1 = \frac{k_3}{k_5}$. When $k_5 >>> k_3$, equation 7 becomes identical to the rate expression of equation 4 for conventional Michealis - Menten kinetics with competitive product inhibition.

In this mechanism, glucose is released first from the enzyme - substrate complex, leaving the enzyme - galactose complex which subsequently releases the galactose molecule. This mechanism also indicates that glucose has no effect on the lactose hydrolysis reaction. These observations were consistent with the mechanisms first postulated by Wallenfels and Wiel [30] and later on by P.J. Deschavanne et al., [48] . It was shown that β - galactosidase - catalyzed reactions proceeded through a chemical intermediate, in addition to the Michealis complex that occur simultaneously with the liberation of the first product of reaction. If lactose is the substrate, a galactosyl -enzyme will be one of the intermediate and glucose would be the first product to be release from the reaction.

## 2.9. Kinetics of ideal enzyme reactor system.

### 2.9.1. Design equation of a plug flow reactor (PFR).

The Michealis - Menten expression for a single substrate hydrolysis reaction in a plug flow reactor was modelized by modifying the rate expression in equation 1 to :

$$v = -\frac{ds}{d\tau} = \frac{V_{max} * S}{K_M + S} \qquad (8)$$

$$\Rightarrow \int d\tau = \int_{So}^{S} -\frac{ds}{\frac{V \max S}{K_M + S}} \tag{9}$$

On integrating equation 9, we obtain

$$\tau = \frac{K_M}{V_{\max}} \ln \frac{So}{S} + \frac{(So - S)}{V_{\max}} \tag{10}$$

where $\tau$ is the residence time of substrate in the reactor.

The degree of conversion (X) of substrate in the reactor can be expressed as :

$$X = \frac{So - S}{So} \tag{11}$$

In the case of an immobilized enzyme reactor, $\tau$ can be represented by the expression :

$$\tau = \frac{V}{F} = \frac{W * Eo}{F} \tag{12}$$

where V is reactional volume in litres, F is the volumetric flow rate (ml/l)

W is the total weight immobilized enzyme, Eo is the enzyme concentration.

By substituting equations 11 and 12 into equation 10, a kinetic design equation for an ideal immobilized enzyme plug flow reactor can be expressed as :

$$\frac{W * Eo}{F} = -\frac{K_M}{V_{\max}} \ln(1 - X) + \frac{XSo}{V_{\max}} \tag{13}$$

### 2.9.2. Product inhibition in a plug flow reactor

Since the biocatalyst to be used in this study is inhibited the hydrolysis product, galactose; it was necessary to model a design equation with competitive product inhibition. By integrating equation 4 and substituting equations 11 and 12 into the obtained result, the kinetic expression with competitive product inhibition for an immobilized enzyme plug flow reactor can be stated as :

$$\frac{W * Eo}{F} = -K_m\left(1+\frac{So}{K_i}\right)\ln(1-X)+\left(1-\frac{K_m}{K_i}\right)So * X \qquad (14)$$

### 2.9.3. Design equation for a continuous stirred tank reactor (CSTR)

For a well - mixed CSTR, the substrate mass balance is expressed as[37] :

$$v * V = q(So - S) \qquad (15)$$

$$\Rightarrow v = \frac{q}{V}(So - S) = \frac{So - S}{\tau} \qquad (16)$$

On substituting equation 16 into equation 1 and then, rearranging the result; we can obtain the design equation for the immobilized enzyme continuous stirred tank reactor to be :

$$\frac{WEo}{F} = \frac{K_M X}{V_{max}(1-X)} + \frac{SoX}{V_{max}} \qquad (17)$$

### 2.9.4. Design equation for CSTR with product inhibition.

The design equation for continuous stirred tank reactor with competitive product inhibition was obtained by substituting equation 16 into equation 4 and then rearranging the result to obtain the expression given below[37, 49] :

$$\frac{W * Eo}{F} = \frac{1}{V_{max}}\left[SoX + \frac{XK_M\left(1+\frac{SoX}{K_i}\right)}{1-X}\right] \qquad (18)$$

### 2.10. Thermal deactivation of immobilized enzyme.

The thermal deactivation of enzyme counteracts the increase in reaction rates at higher temperatures. Amongst the numerous theoretical and mathematical models[44, 50.51, 52] proposed so far, only the model developed by Henley and

Sadana[53] has encompassed most of the more specific deactivation models. An irreversible first order reaction kinetic has been reported to fit the thermal deactivation of lactase; i.e., the activity decays exponentially with time[54] . The reaction rate coupled with deactivation, can be expressed in terms of residual activity, if the rate of reaction is faster than enzyme deactivation rate [47,52]

$$E \xrightarrow{k_d} E_d \qquad (19)$$

where E is the active enzyme, $E_d$ is deactivated enzyme,

$K_d$ is the deactivation constant.

This process leads to an exponential decay with enzymatic activity expressed as

$$A/A_o = e^{-k_d t} \qquad (20)$$

where A is the total activity at time, t. Ao is the total activity at time, t = 0. When there is enzyme deactivation this equation can be substituted in equation 14 to obtain the equation below :

$$\frac{WEo}{F}\exp(-kd * t) = -Km\left(1+\frac{So}{Ki}\right)\ln(1-X)+\left(1-\frac{Km}{Ki}\right)SoX \qquad (21)$$

where $K_d$ is the deactivation constant of the immobilized enzyme.

# III. MATERIALS AND METHODS.

## 3.1. MATERIALS.

### 3.1.1 Experimental equipments

- Two reactors; a continuous stirred tank reactor and a plug flow reactor, both made of plexiglass were inserted into the experimental circuit.

- A peristaltic pump used for pumping the substrate into the reactors.

- A Constant temperature bath on which an immersion heater is attached.

- Flow meter to regulate the substrate feeding rate of substrate into the CSTR

- A basket - type stirrer for the CSTR.

- Five (5) temperature probes connected to a temperature recorder

- A tubular polysulfone membrane obtained from Osmonics Inc., was inserted into the UF unit.

### 3.1.2. Equipment for analyzing samples and products.

- High Performance Liquid Chromatography was used to analyzed sugars (lactose, galactose, and glucose). This HPLC had a Gilson refractive index detector, model 132, a Gilson automatic sample injector, model 231 and a Gilson dilutor, model 401. The pump utilized was a waters associate pump, model 6000A. Water was employed as the eluent at a flow rate of 1 ml/min. The column heating apparatus was from Man-Tech Inc., model 7980 and the analyzing column was a Bio-Rad, Aminex HPX-87C Column.This column was excellent in separating a variety of carbohydrates (sugars). The operating temperature of the column was 80°C. The quantity of sample utilized each time was 20 μl.

- VARIAN Instrument, model AA - 1275 series, Atomic Absorption spectrophotometer was used to analyzed cations concentration in the substrate (Cheese industrial effluent).

- A Nicolet 510 P, FT - IR Spectrometer was used to record and study the interactions between the sugar-cation complexes.

- The Hewlett Packard Diode - Array Spectrophotometer, model HP 8452A, with an operating software, HP 89531A MS-DOS(R), UV/VIS, was used to analyzed ONPG and ONP concentration required for determining the initial activity of the enzyme as well as the operating conditions of the immobilized enzymes.

### 3.1.3. CHEMICALS

The chemicals used in this research are listed below;

| CHEMICALS | DESCRIPTION | SUPPLIERS |
|---|---|---|
| Calcium Hydroxide | $Ca(OH)_2$ | Aldrich |
| Glutaraldehyde | $HCO(CH_2)_3CHO$ | Aldrich |
| Lactic Acid | 85% | Aldrich |
| Magnesium sulfate | $MgSO_4$ | Aldrich |
| Polyethylenimine | PEI | Aldrich |
| Potassium hydroxide | KOH | Aldrich |
| Potassium sorbate | $CH_3(CH)_4CO_2K$ | Aldrich |
| Sodium hydroxide | NaOH | Aldrich |
| 2-Nitrophenyl-B-D-galactopyranoside | ONPG | Aldrich |
| 2 - Nitrophenol(ONP) | $O_2NC_6H_4OH$ | Aldrich |
| Calcium Standard | $Ca^{++}$ | BDH Inc. |
| Magnesium | $Mg^{++}$ | BDH Inc |
| Potassium | $K^+$ | BDH Inc |
| Sodium | $Na^+$ | BDH Inc |
| Sodium acetate | $CH_3COONa$ | Canadawide |
| Sodium phosphate(monobasic) | $NaH_2PO_4.2H_2O$ | Canadawide |
| Sodium phosphate(dibasic) | $Na_2HPO_4.12H_2O$ | Canadawide |
| Acetic acid (Glacial) | $C_2H_4O_2$ | Sigma Chemicals |
| Galactose | $C_6H_{12}O_6$ | Sigma Chemicals |
| Glucose | $C_6H_{12}O_6$ | Sigma Chemicals |
| Lactose | $C_{12}H_{22}O_{11}.H_2O$ | Sigma Chemicals |

# III.2 EXPERIMENTAL PROCEDURE .

## 3.2.1. Immobilization method.

### 3.2.1.1. Enzyme support

Two types of biocatalyst carriers were used in this research. The granular diatomaceous earth (GDE) was obtained from Eagle Pitcher Minerals Inc. Michigan, U.S.A. This porous GDE has good mechanical strength, and stability on exposure to heat and to acid or basic solutions. The particle diameter was between 0.74 - 0.85 mm and the pore size was 22 mesh size U.S. standard. The other bio-catalyst carrier, Celite R - 640 was obtained from Celite Corporation P.O. Box 519 Lompoc, CA 93438-0519, U.S.A. The celite R-640 carrier was made of rigid inorganic materials and was in pellet form. This carrier had a very good chemical and thermal stability. The mean pore diameter was 0.03 μm and the surface area was 61 $m^2/g$. In order to select which biocatalyst carrier was to be used in this research , immobilisation efficiency, enzyme loading per gram of dry weight support, mechanical and thermal stability were investigated.

### 3.2.1.2. Lactase

Two kinds of enzymes were tested; β - galactosidase ( EC 3.2.1.23) from Aspergillus oryzae was purchased from Sigma Chemicals and had an initial activity of 4.4 units/mg solid with lactose or 4.5 units/mg solid with ONPG at 30 °C and pH 4.5. The other enzyme, Lactozyme 3000 L, type HP obtained from the

Novo Industries, CT, U.S.A. had an initial activity of 3000 LU (lactose unit) at 37 °C and pH 6.5.

### 3.2.1.3. Determination of initial enzyme activity in ONPG units.

In order to determine the initial activity of these enzymes, a standard curve for orthonitrophenol (ONP) was made from various initial concentration of ONP [ see Appendix 2]. Equal amounts of soluble enzymes were placed in five test tubes containing different concentrations of ortonitrophenyl-β-galactopyranoside (ONPG), a colourless substrate which is hydrolyzed by lactase into galactose and the yellow-coloured ortonitrophenol (ONP). The reaction was stopped after 2 minutes by adding 1 ml of 1M $Na_2CO_3$ to each test tube.

### 3.2.1.4 Immobilization

The immobilization method used in this research was invented by Chiang , J. P. and Lantero, O. J. Jr [41] and published in the European patent office.

### 1. Treatment of enzyme support.

39 g GDE was washed thoroughly with distilled water or di-ionized water until the supernatant was clear. After the GDE had settled, the supernatant liquid was decanted. The diatomaceous earth was suction deaerated and the residual water decanted.

## 2. Immobilization of enzymes.

500ml of 0.2% w/v aqueous solution polyethylenimine (PEI) with pH 9.8 was added to the carrier in a 1L Erlenmeyer flask and the flask was placed in a microprocessor bath. It was agitated gently at a speed of 150 rpm for 4 hours. Excess PEI was decanted and the treated carrier washed thoroughly with water. The pendant amine groups of the polymer attached to the carrier was derivatized by adding 500ml of 0.5% w/v glutaraldehyde made up 0.05M $NaHCO_3$, in pH 8.2. The flask was agitated gently for 2 hours at room temperature. The excess glutaraldehyde was decanted and the treated carrier washed with di-ionized water to remove unreacted glutaraldehyde. The result was a carrier comprising diatomaceous earth, polyethylenimine, and glutaraldehyde. One gram of lactase from Aspergillius oryzae was dissolved in 500 ml of 0.02 M phosphate buffer adjusted to pH 7.0 and then, added to the derivatized support. It was then agitated gently for 4 hours at room temperature.

The residual buffer solution was decanted and stored in order to determine the residual solution activity. The IME was washed with copious amount of de-ionized water. The washed IME was stored in Potassium Sorbate solution and kept in a freezer until utilisation was required.

### 3.2.1.5 Residual enzyme activity

The residual immobilization solution was reacted with ONPG and the activity, $E_1$ in ONPG Units was obtained. The immobilization efficiency was calculated by using the formula :

$$\text{Immobilization Efficiency, } \alpha = \left[\frac{Eo - E1}{Eo}\right] * 100 \qquad (22)$$

where Eo = Initial enzyme activity and $E_1$ = residual enzyme activity

### 3.2.1.6 Determination of immobilized enzyme thermal stability.

Two (2) g of *A. oryzae* immobilized on GDE was placed in 5 ml 2mM ONPG and adjusted to a pH 4.5 with acetate buffer. 1 ml of solution was removed each time and placed in 1ml 0.12N NaOH to stop the enzymatic reaction. The amount of ONP formed was measured at 420 nm. This was repeated for other temperatures(40, 50, 55 $^{o}$C).

### 3.2.1.7 Determination of immobilized enzyme mechanical stability.

6 g of IME (*A.oryzae* on GDE) was placed in the CSTR containing 50 ml of water. The stirring speed ($\varphi$) was varied from 1 rps -3 rps. After every 15 minutes, 1 ml of solution was removed and reacted with 1 ml of ONPG adjusted to pH 4.5 with an acetate buffer. This reaction was stopped after 2 minutes with NaOH . The amount of ONP formed was measured at 420 nm.

## 3.2.2. KINETIC STUDIES.

Kinetic studies were performed on pure lactose without cations and then, in the presence of cations. The kinetic studies in the presence of cations were carried out with individual cations as well as a combination of the three principal cations found in cheese industrial effluent.

### 3.2.2.1 Kinetic studies without cations.

#### 3.2.2.1.1 Effect of temperature.

The effect of temperature on lactose hydrolysis kinetics was performed with a 2 % (w/v) lactose solution in a temperature range of 30 °C - 60 °C. 6 g of immobilized enzymes was placed in a 100 ml flask containing 25 ml lactose solution whose pH has been adjusted to 4.5 with an acetate buffer. The flask was placed in a constant temperature microprocessor bath and allowed to shake gently. The reaction was left to continue in a stirred batch reactor (flask) for 180 minutes during which samples were taken every 15 minutes for analysis.

The samples were filtered with 0.45 μm nylon 66 membrane filter and immediately heat-treated in about 80 °C hot water bath to stop the enzymatic reaction. An HPLC was used to analyzed the galactose, glucose, lactose, and oligosaccharide

present in the sample. The degree of hydrolysis or conversion (X ) was calculated using the equation below:

$$X = \left[ \frac{C_{glu\,cos\,e} + C_{galactose}}{C_{lactose} + C_{glu\,cos\,e} + C_{galactose}} \right] * 100 \qquad (23)$$

where C is the concentration of lactose, glucose and galactose present in the analyzed sample.

### 3.2.2.1.2 Effect of various initial lactose concentration.

The influence of various initial lactose concentration on the rate of lactose hydrolysis by immobilized enzymes was investigated experimentally at a constant temperature of 50 °C . An acetate buffer was used to stabilized the pH at 4.5. An appropriate amount of IME with an enzyme loading of 26,3 mg/ g dry wt. support were placed in 100 ml flask containing 25 ml of different concentrations of lactose solutions. These flasks were placed in a constant temperature microprocessor bath and allowed to shake gently. The concentration of lactose varied from 0.5 % (w/v) to 5 % (w/v). The reaction was performed in a stirred batch reactor for 180 minutes and samples were taken every 15 minutes for analysis. The samples were filtered with 0.45 μm nylon 66 membrane filter and immediately heat-treated in about 80 °C hot water bath to stop the enzymatic reaction. An HPLC was used to analyzed the galactose, glucose. lactose and oligosaccharide present in the sample. The degree of conversion (X ) was calculated using equation 23.

### 3.2.2.2. Kinetic studies in the presence of cations.

#### 3.2.2.2.1. The case of single cation

A similar experiment as in 3.2.2.1.1 above was performed with 2% (w/v) lactose in the presence four individual cations namely, 0.1M NaCl, 0.1M KCl, 2 mM $CaSO_4$, and 2 mM $MgCl_2$.

#### 3.2.2.2.2. The case of a combination of three cations.

A similar experiment as in 3.2.2.1.1 above was performed in the presence of a combination of three cations namely; $K^+$ , $Na^+$, $Ca^{++}$. Their concentration ranges were as follows :

| | | |
|---|---|---|
| NaCl | : | 0.05 M - 0.1 M |
| KCl | : | 0.05 M - 0.1 M |
| $CaSO_4$ | : | 0.5 mM - 2 mM |

### 3.2.3. Experimental circuit.

#### 3.2.3.1 Ultrafiltration (UF) unit

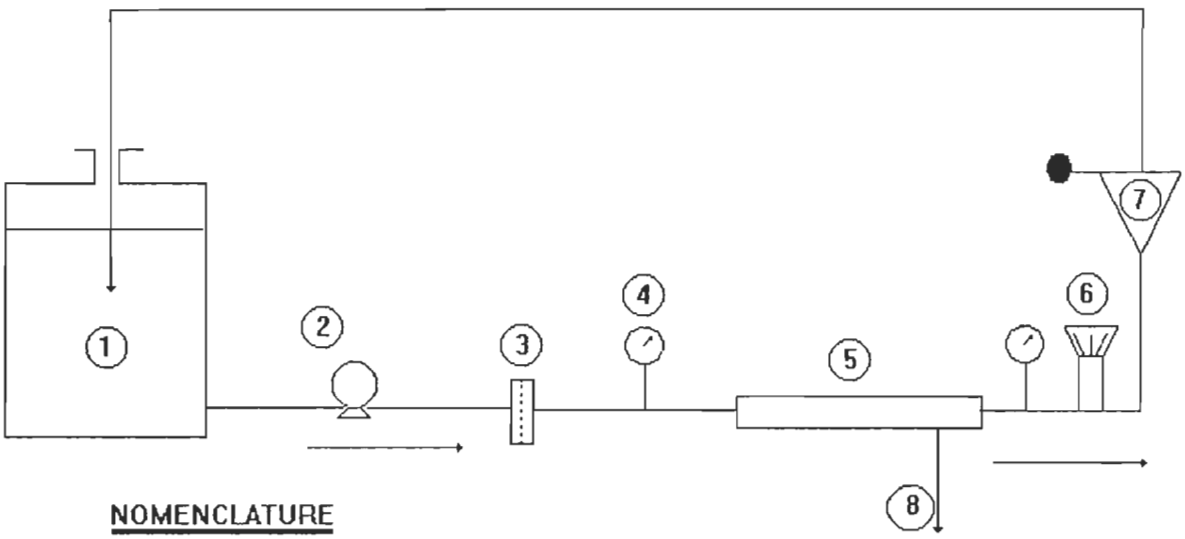
Cheese effluent obtained from the cheese plant was placed in a 20 liters plastic container and a 1hp pump was used to pump the substrate through a catridge filter to remove extremely large particles before sending it to the UF unit. A tubular membrane obtained from Osmonics Inc., U.S.A. was used to separate the effluent into whey protein concentrate (retentate) and whey permeate. Most the salts or

cations were also removed during this process The feeding rate was about 70 ml/s and the pressure applied was about 350 kpa. The ultrafiltrate ( permeate) was stored in a freezer and ready to be used in the bioreactors. Figure 3 illustrate the diagram of the ultrafiltration unit.

### 3.2.3.2 Hydrolysis of cheese industrial effluent in bioreactors.

The experimental circuits as shown in figure 4 and figure 5 were thoroughly flushed with dionized water containing a few drops of 5.25 % sodium hypochlorite solution to desinfect the system from any microbial contamination prior to performing the experiments.The continuous stirred tank reactor (CSTR) and the plug flow tubular reactor (PFTR) were constructed of plexiglass. A proportionate amount of immobilized enzymes depending on the reaction volume of each reactor was placed in the CSTR and PFTR. A variable speed peristaltic pump was used to pump the 4 °C whey permeate through a spiral stainless steel tube-type heat exchanger, placed in a constant temperature bath. Here, the feed was warmed to the required operating temperature. The feed was then pumped at a constant flow rate of 15 ml/min or 40 ml/min into the CSTR or the PFTR respectively. The reactors were maintained at isothermal condition of 50 °C as the substrate by a continuous stream of hot water circulating through the jacket of the reactor and the constant temperature water bath. Medical grade tygon tubing and stainless steel piping were employed in the experimental circuit. A polyethylene filter with a mesh

FIGURE 3 : ULTRAFILTRATION UNIT




NOMENCLATURE

1- Substrate Container

2 - pump

3 - Prefilter (5 µm)

4 - Manometer

5 - UF membrane

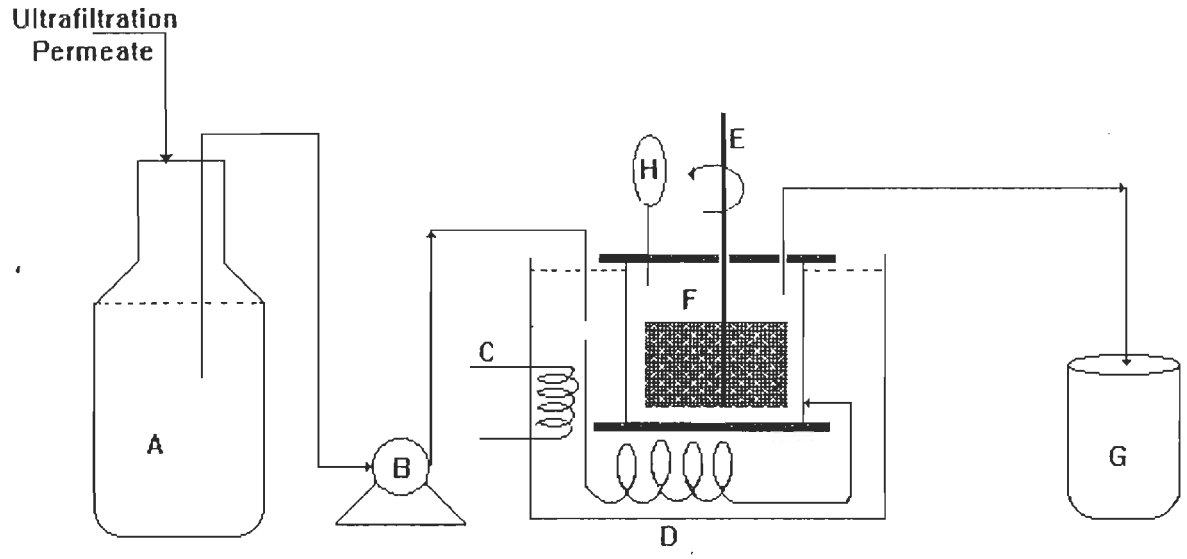
6 - Pressure regulator

7 - Flow meter

8 - Whey permeate

size of 408 μm was used to support the immobilized enzymes bed in the plug flow tubular reactor. A similar type of screen was placed at the exit of the continuous stirred tank reactor to prevent immobilized enzymes from going into the product stream. A high flow rate of feed solution was used to eliminate diffusion limitation.The outlet stream of the reactors were collected and tested for the presence of glucose, galactose, lactose, and oligosaccharides.The degree of hydrolysis, X (%) was calculated using the expression in equation 23, where C is the concentration in the hydrolyzed permeate collected at the reactor's exit.

FIGURE 4 : EXPERIMENTAL CIRCUIT.




NOMENCLATURE

A : Substrate Container

B : Peristaltic Pump

C : Heater

D : Thermostable Bath

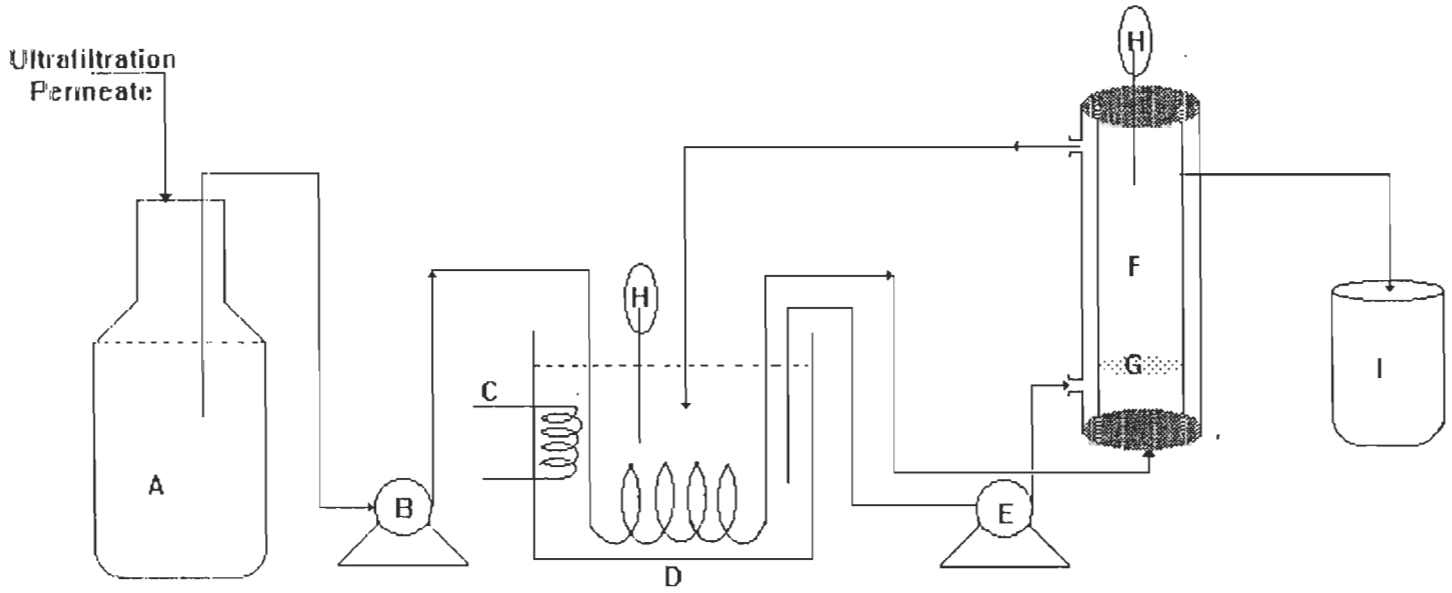
E : Basket - type stirrer

F : Continuous Stirred Tank Reactor

G : Product Container

H : Thermistor thermometers

FIGURE 5 : EXPERIMENTAL CIRCUIT.




NOMENCLATURE

A : Substrate Container

B : Peristaltic Pump

C : Heater

D : Thermostable Bath

E : Recirculation Pump

F : Plug Flow Reactor

G : Filter

H : Thermistor thermometers

I : Product Container

### 3.2.4. Biochemical and chemical oxygen demand analysis.

The Biochemical oxygen demand ($BOD_5$) and chemical oxygen demand (COD) of the sampled effluents and the hydrolyzed effluents were measured using the dissolve oxygen meter at the pulp and paper research center (CRPP) of Université du Québec à Trois - Rivières.

### 3.2.5. Spectroscopic study of lactose - cation complexes.

The D - lactose - metal ion complexes were prepared by mixing separately 1 ml of 0.1 M of hydrated metal halides (KCl, NaCl, and $CaCl_2$) in water to 1 ml solution of 0.1M D - lactose in water. The mixture was heated for 10 minutes at 70 °C and then cooled to room temperature. Four drops of each mixture were placed on a $CaF_2$ window and allow to evaporate at room temperature and low pressure to obtain a uniform film of the complex precipitate on the cell. This precipitate was an adduct of the type M(D-lactose)X.$nH_2O$, where M represents the cations K or Na, and X represents the anion (Cl). In the case of calcium chloride, the adduct was of the type, Ca(D-lactose)$Cl_2$.$nH_2O$. It should be mentioned here that all the chemicals were reagent grade and were used without further purification. A Nicolet type FT-IR spectrophotometer was used to record the infrared spectra. This procedure was repeated for different concentrations( o.1M, 0.5M and 1M) of metal ions. Infrared spectra were recorded in the range of 1800 $cm^{-1}$ to 1000 $cm^{-1}$ were studied.

# IV. RESULTS.

## 4.1. Immobilization results.

### 4.1.1. Immobilization efficiency

During the immobilization process, the erlenmeyer flask containing the enzyme and treated support was placed in a microprocessor shaker bath. The agitating speed,$\omega$ was varied from 60 rpm - 175 rpm. The results obtained are illustrated in Table 5. By plotting immobilization efficiency against agitating speed, the curve in figure 6 was obtained.

### 4.1.2. Enzyme support

The immobilization efficiency and enzyme loading per gram of dry weight support obtained with the optimum immobilization agitating speed of 150 rpm for the lactase, Aspergillus oryzae are summarized in table 6. This result was used in determining which enzyme carrier would be used in this research.

### 4.1.3. Initial activity

The results obtained by measuring the absorbance at 420 nm was used to plot graph of the absorbance as a function of ONPG concentration. From this graph the initial activity of the two enzymes were determined in ONPG unit. One activity unit is defined as the amount of micromoles of ONPG hydrolyzed per minute per mg of enzyme. The initial activity of Aspergellus oryzae was found to be 4,66 units/mg enzyme while that of lactozyme was 2480 units/mg enzyme.

**Table 5**

**Immobilization of *Aspergillus Oryzae*.**

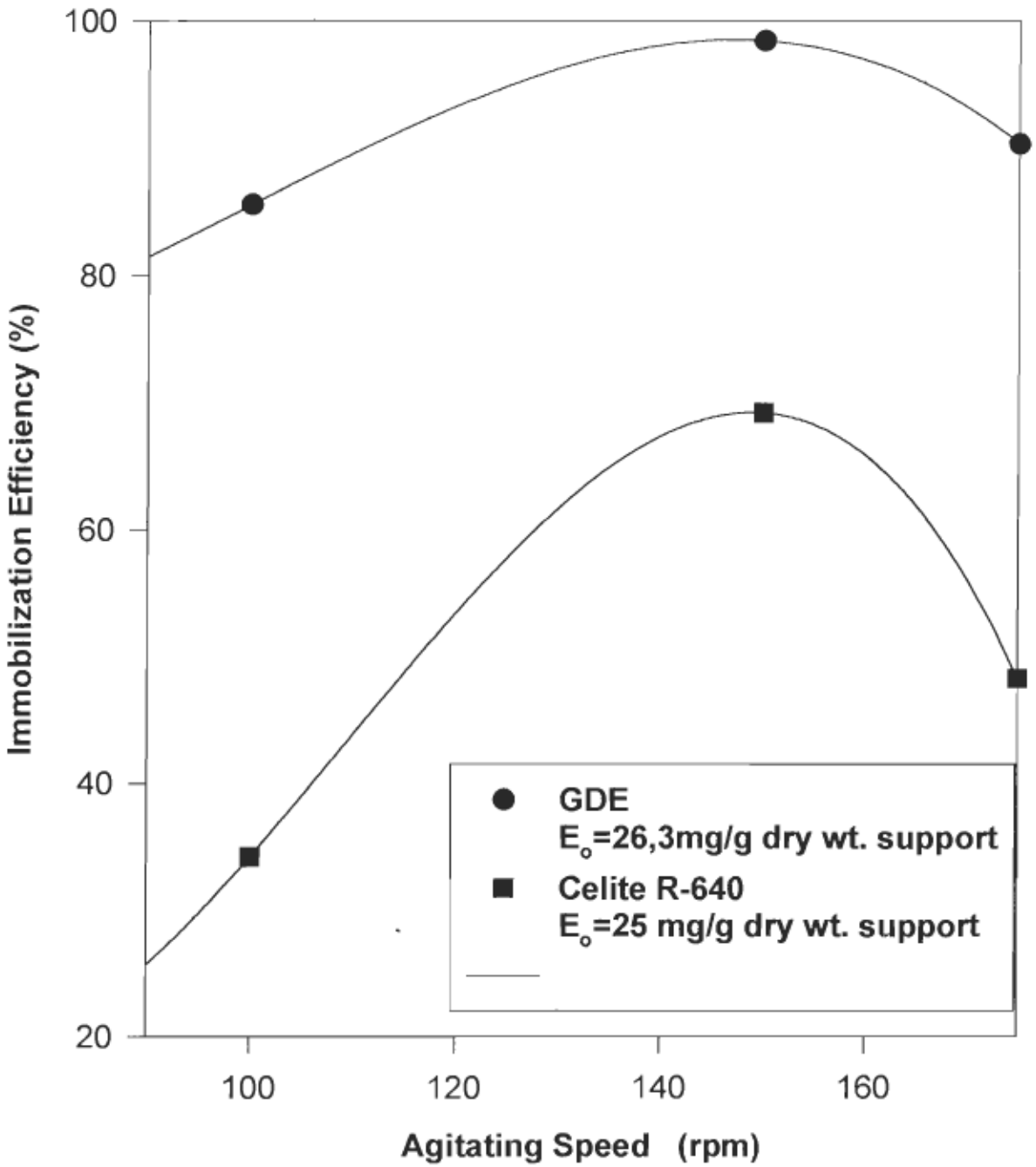
| | Immobilization | Efficiency, ε (%) |
|---|---|---|
| **Agitating Speed (rpm)** | **GDE** | **Celite R - 640** |
| 60 | 72.7 | 20.52 |
| 100 | 85.63 | 34.2 |
| 150 | 98.4 | 69.2 |
| 175 | 90.35 | 48.3 |

**Table 6.**

**Carrier Selection for the Enzyme ; *Aspergillus oryzae*.**

| Support | Enzyme Loading mg/g dry wt. support | Initial Activity ONPG units | Immobilization Efficiency( % ) |
|---|---|---|---|
| Celite R - 640 Diatomaceous Earth(GDE) | 25.0 | 4.66 | 69.2 |
| | 26.3 | 4.66 | 98.4 |

**Figure 6 : Effect of Agitating speed on Immobilization Efficiency using different Biocatalyst Supports.**



### 4.1.4. Thermal stability of immobilized enzyme.

The experimental data collected for thermal stability of immobilized A. oryzae in the temperature range studied were employed in plotting the graphs of [ONP] formed as a function of time, t, illustrated in figure 7. This experiment was necessary in order to determine the optimum operational temperature of the immobilized enzyme.

### 1.5. Mechanical stability of immobilized enzyme

Figure 8 represents the graph of [ONP] formed as a function of time, t, obtained from the immobilized enzyme mechanical stability experiment. The purpose of this particular experiment was to determine the optimum agitating speed of the agitator when performing hydrolysis reaction with IME in CSTR.

## 4.2. Kinetic results.

### 4.2.1. Kinetic of lactose hydrolysis without cations.

#### 4.2.1.1. Temperature influences.

The experimental data for pure lactose hydrolysis for temperatures ranging from 30 °C to 60 °C is given in appendix 3 . In order to determine the reaction rates, the best equation fits for the plots of glucose concentration produced during the hydrolysis reaction as a function of time as shown in figure 9 were determined. The slopes of these curves gave the reaction rates.

## Figure 7 : Thermal Stability of Immobilized A. Oryzae.

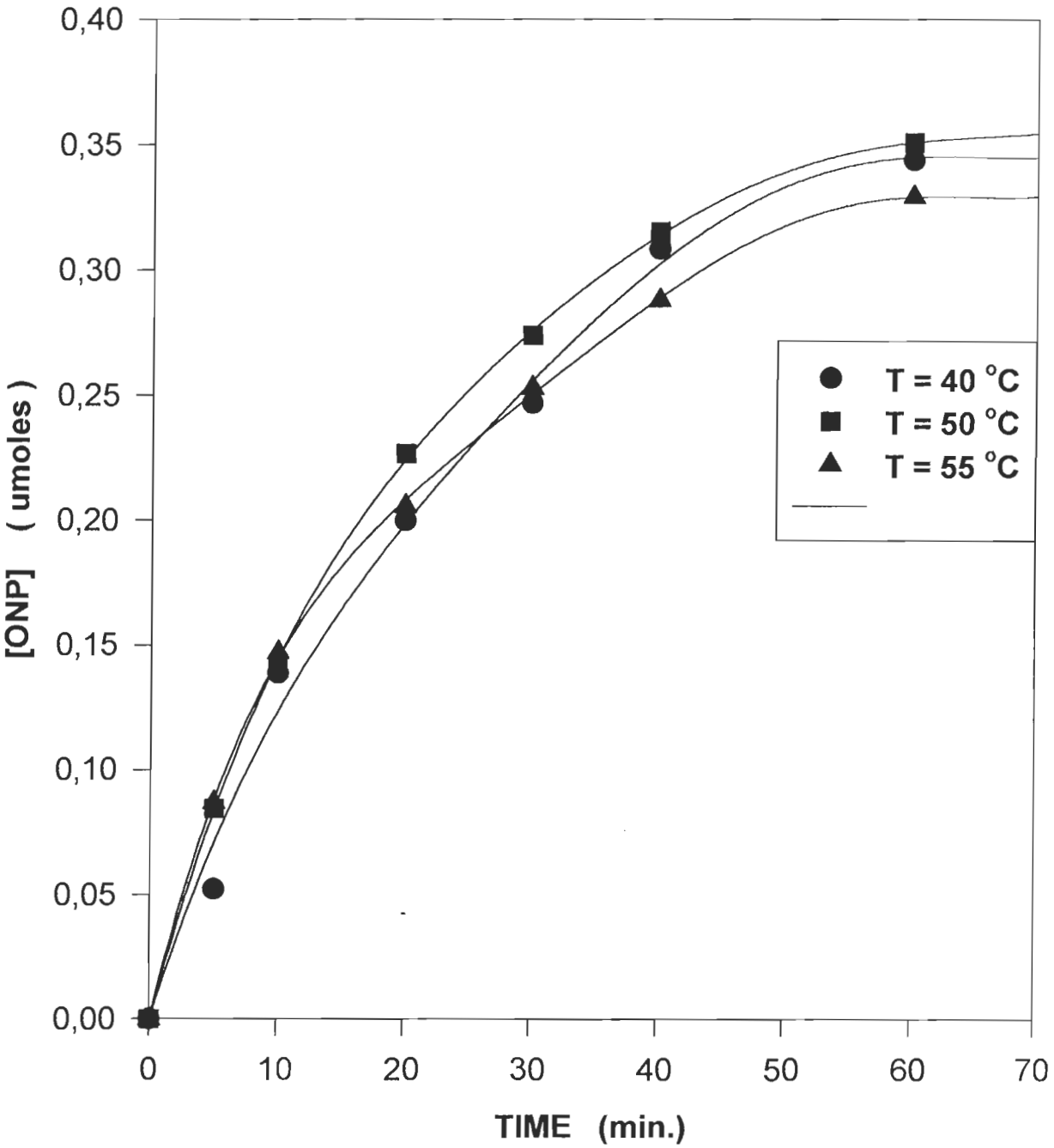


**Figure 8 : Mechanical Stability of Immobilized A. Oryzae in CSTR.**

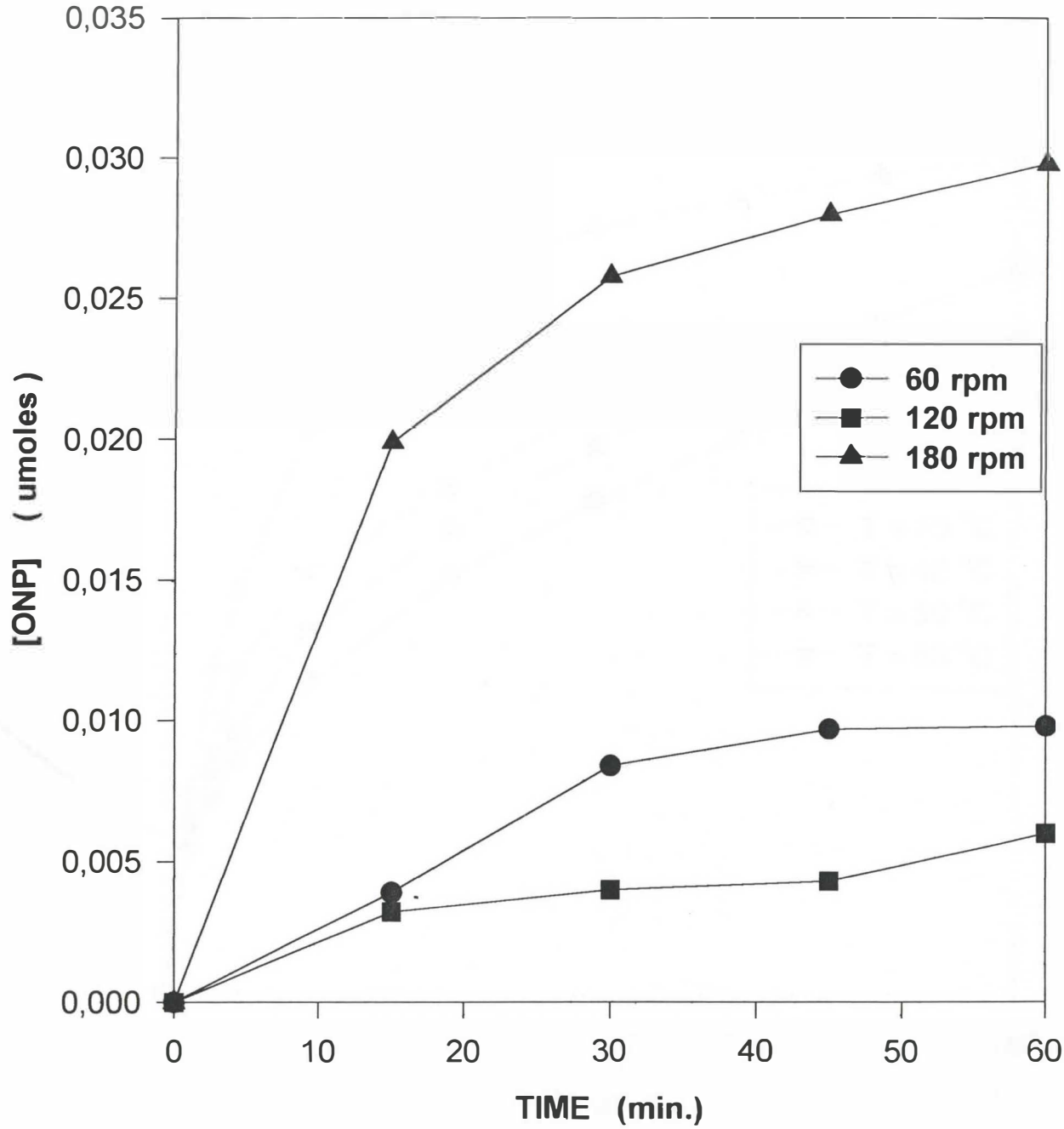
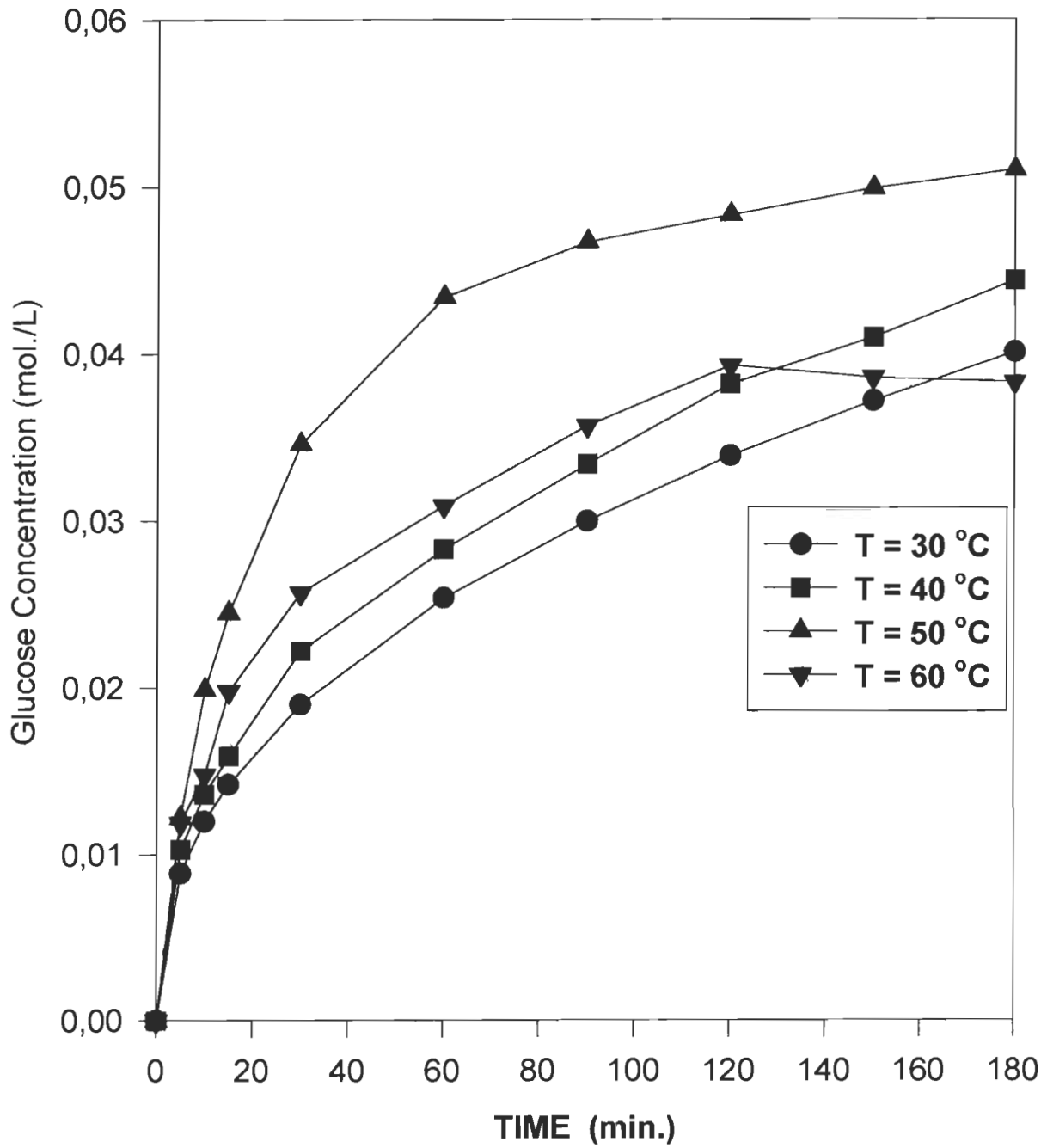


**Figure 9 : Kinetic Hydrolysis of 2% (w/v) Lactose.**



By plotting the obtained reaction rates , v, as a function of the initial lactose concentration, [S], as shown figure 10 and applying a non - linear computer regression method to the Michealis - Menten type kinetic with competitive product inhibition represented by equation 4, the kinetic parameters (apparent maximum rate of reaction, $V_{max.app}$, and the apparent Michealis-menten kinetic constant, $K_{m.app.}$) were obtained. The results are summarized in table 7.

#### 4.2.1.2 The effect of initial lactose concentration.

The experimental data obtained were used to make the plots of initial lactose concentration as a function of time shown in figure 11. Initial rates of reaction, R, were obtained by doing the first derivative of the best equation fit of each of the curves in figure 11. The values of the reaction rates obtained were plotted against lactose concentration to obtained the kinetic parameters as indicated in equation 4. These kinetic parameters are represented as $V_{m.app.}$ and $K_{m.app.}$. The kinetic parameters obtained are summarized in table 8.

**Table 7.**

**Effect of temperature on kinetic parameters.**

| TEMPERATURE<br>° C | Vmax.<br>mmol./L.min.$g_{IME}$ | Km app.<br>mmol./ L |
|---|---|---|
| 30 | 2.50 | 20.82 |
| 40 | 3.13 | 20.64 |
| 50 | 6.11 | 46.52 |
| 55 | 7.56 | 39.96 |
| 60 | 10.97 | 53.30 |

**Table 8 :**

**Effect of Initial lactose Concentration on Kinetic Parameters.**

| Lactose Concentration<br>% ( w/v ) | Km.app.<br>mmol. $L^{-1}$ | Vmax.<br>mmol.$L^{-1}$.$min^{-1}$. $g_{IME}^{-1}$ |
|---|---|---|
| 0.5 | 18 | 0.572 |
| 1 | 26 | 1.049 |
| 2 | 46.52 | 1.89 |
| 3 | 42.37 | 2.56 |
| 4 | 63.23 | 3.08 |
| 5 | 110.5 | 3.55 |

**Figure 10 : Effect of Temperature on the rate of Lactose hydrolysis reaction**

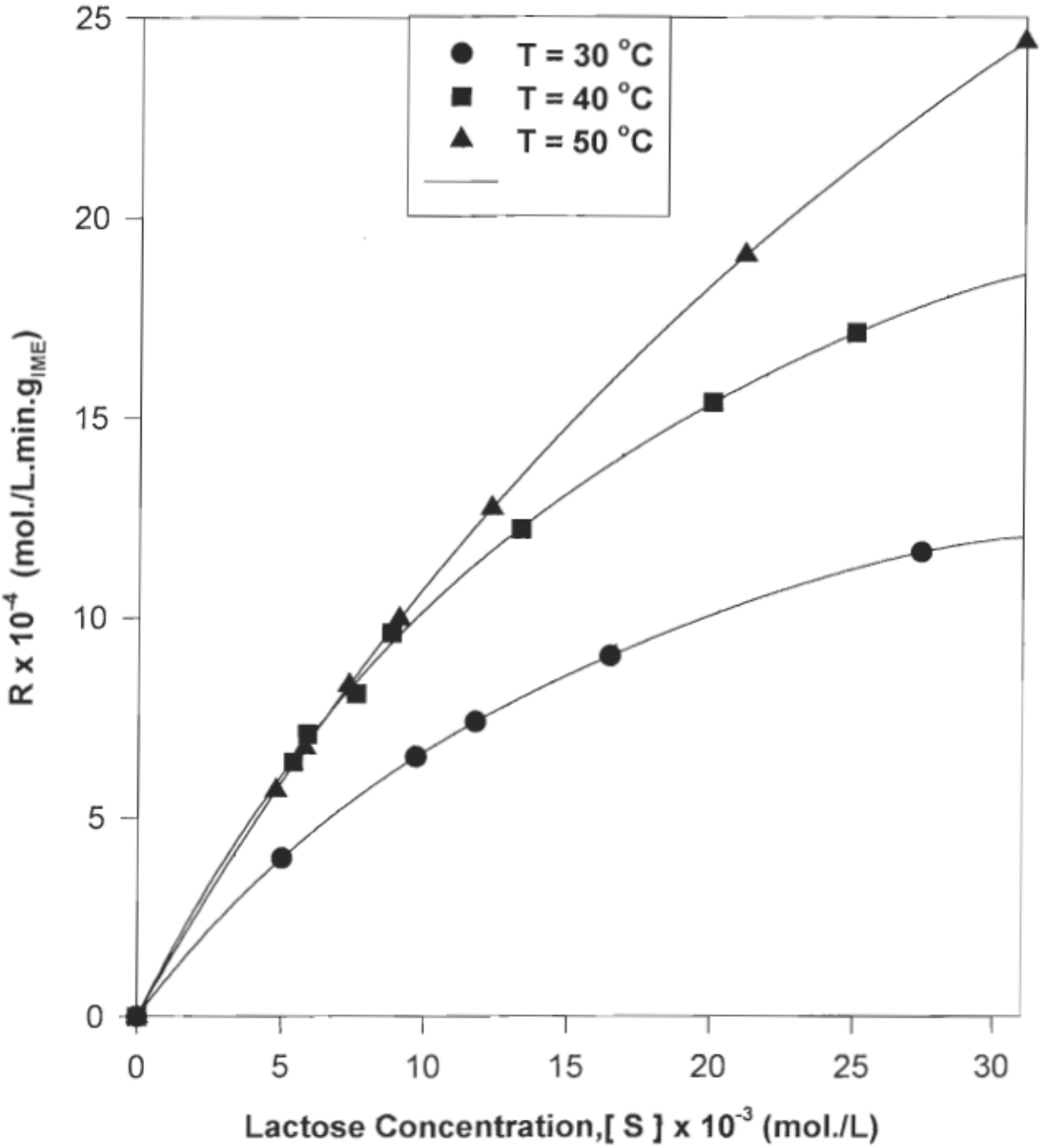
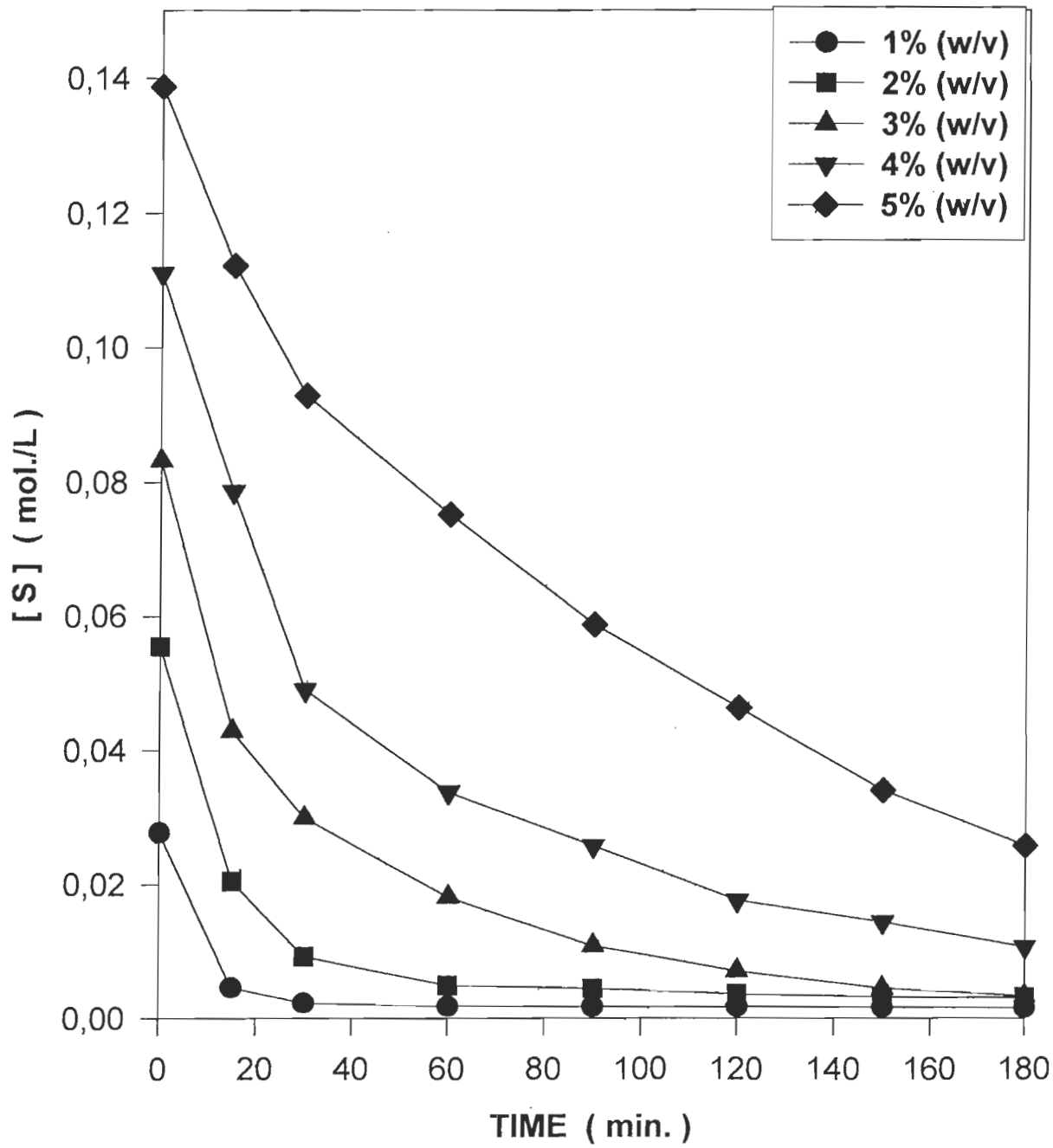


**Figure 11 : Hydrolysis of various initial Lactose Concentration**



### 4.2.2. Kinetic of lactose hydrolysis in the presence of cations

#### 4.2.2.1. The case single cation.

The experimental data obtained from the hydrolysis of lactose in the presence of single cations at a temperature of 50 °C are listed in Appendix 4.

Cations were tested individually to investigate the effect of each individual cation on the kinetic parameters. The obtained results were compared to those obtained from a real cheese factory effluent hydrolyzed in the bioreactors.as well as those obtained from the enzymatic hydrolysis of lactose in the absence of cations. The Michealis - Menten rate model in equation 4 was used to obtained the kinetic parameters given in table 9 for each individual cation. The results obtained from the degree of conversion as a function of time, t, are shown in figure 12.

#### 4.2.2.2. The case of a combination of three (3) cations.

The results obtained from a combination of the three principal cations present in cheese industrial effluent are reported here. By maintaining the concentration of $[Na^+] / [K^+] = 1$ and varying the concentration of $Ca^{2+}$, the result of the kinetic parameters obtained are given in table 10. Similarly, by keeping the concentration of $[Na^+] / [Ca^{2+}] = 100$ and varying the concentration of $K^+$ the results obtained for the kinetic parameters are represented in table 11.

Finally, the results of the kinetic parameters obtained by varying the concentration of $Na^+$ while maintaining $[K^+] / [Ca^{2+}] = 100$ are shown in table 12.

**Table 9**

**Effect single cations on kinetic parameters**

| Cation & Concentration mol./L | Vmax. mmol./L.min.$g_{IME}$ | Km app. mmol./ L |
|---|---|---|
| 0.1M $Na^{+}$ | 2.05 | 45.55 |
| 0.1M $K^{+}$ | 3.59 | 83.00 |
| 0.002M $Ca^{++}$ | 6.34 | 38.87 |
| 0.002M $Mg^{++}$ | 9.68 | 35.59 |
| **No Cation** | **6.11** | **46.52** |

**Table10.**

**Effect of [$Ca^{++}$] on kinetic parameters, keeping [Na+]/[K+] = 1**

| [$Ca^{++}$] Concentration Mol./ L | Vmax. mmol./L.min.gIME | KM app. mmol./ L |
|---|---|---|
| 0.0005 | 2.72 | 37.46 |
| 0.001 | 3.09 | 17.55 |
| 0.002 | 5.13 | 17.20 |

**Table 11**

**Effect [$K^+$] on kinetic parameters, keeping [$Na^+$]/[$Ca^{++}$] = 100**

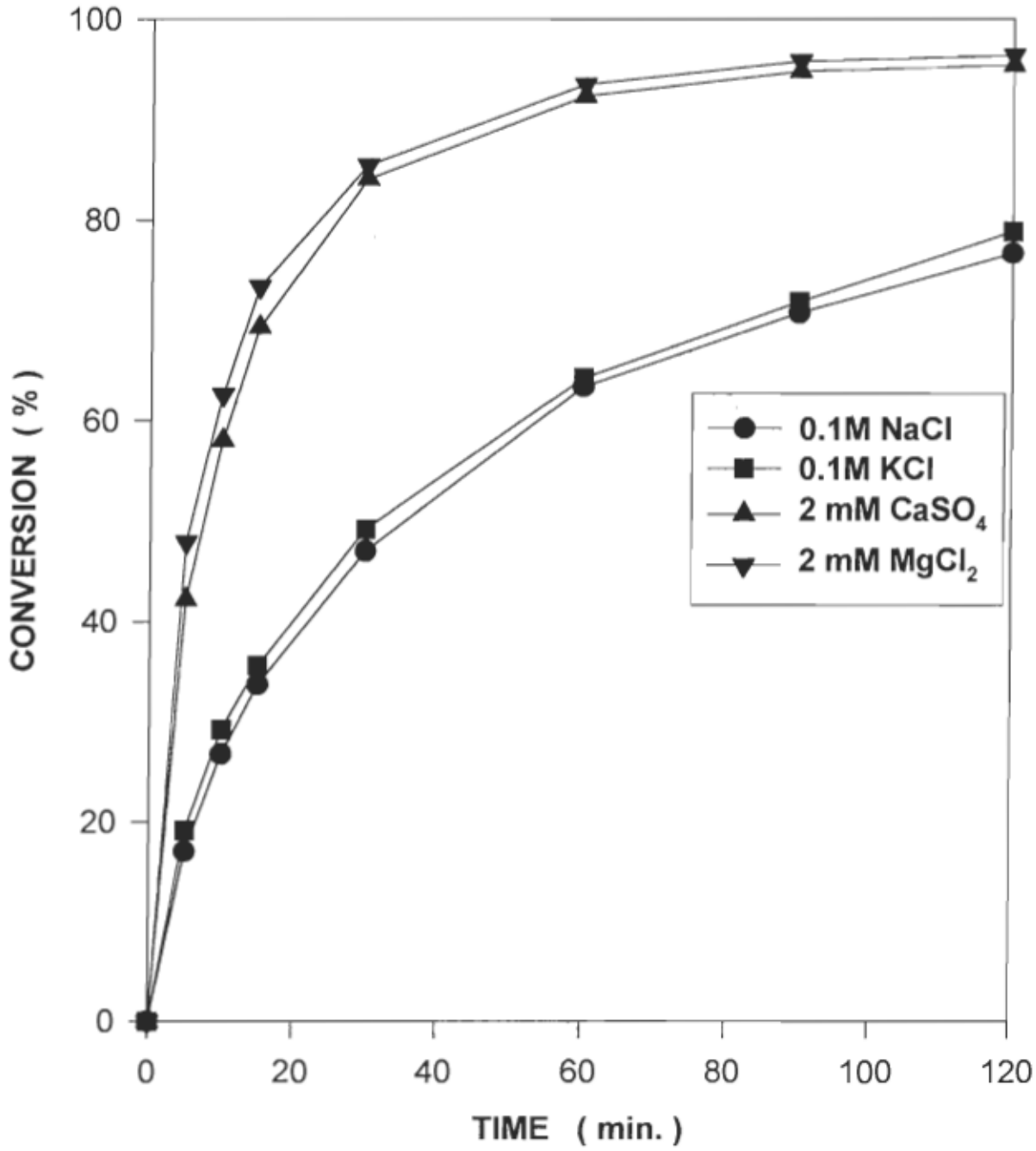
| $K^+$ Concentration<br>Mol./ L | Vmax.<br>mmol./L.min.$g_{IME}$ | Km app.<br>mmol./ L |
|---|---|---|
| 0.05 | 2.85 | 7.222 |
| 0.075 | 4.89 | 46.19 |
| 0.100 | 3.47 | 40.83 |

**Table 12**

**Effect of [$Na^+$] on kinetic parameters, keeping [$K^+$]/[$Ca^{++}$] = 100**

| $Na^+$ Concentration<br>Mol./ L | Vmax.<br>mmol./L.min.$g_{IME}$ | Km app.<br>mmol./ L |
|---|---|---|
| 0.05 | 2.85 | 7.222 |
| 0.075 | 3.17 | 26.69 |
| 0.100 | 3.60 | 53.40 |

**Figure 12 : Effect of Single Cations on the Hydrolysis of Lactose at 50 °C**



## 4.3. FT-IR spectroscopic studies on lactose-cation complexes.

The results of infrared spectra obtained for various concentrations of the chlorides of potassium, sodium and calcium upon complexation with D-lactose are shown in figures 13, 14, and 15 respectively. The absorbance obtained at 1650 $cm^{-1}$ where a strong and broad regularly increasing absorption bands were observed in the region studied in this research as well as the calculated coefficient of absorption for the free D-lactose and Ca(D-lactose)$Cl_2$.$nH_2O$ complexes are presented in table 13.

## 4.4. Hydrolysis of cheese Industrial effluent in bioreactors .

The samples obtained from Agropur's cheese factory at Notre-Dame du Bon Conseil were tested in the two types of bioreactors; CSTR and PFTR. Sample A was cheese production plant effluent or the wastewater treatment plant affluent while Sample B Waste water treatment plant effluent.

An analysis of the ultrafiltration permeate of these samples is given in table14. In order to compare the two reactors, the same residence time of substrate was applied to both reactors. A plot of the degree of hydrolysis Vs time, t, is shown in figures 16 and 17 for samples A and B respectively.

## 4.5. The results of the biological and chemical oxygen demand.

The results given in table 15 were obtained by measuring the biochemical and chemical oxygen demand of the sampled effluents and the hydrolyzed effluents using the dissolve oxygen meter at pulp and paper research centre of the university of Queébec at Trois - Rivières.

**Table 13 :**

**FT - IR study of free lactose and Ca - lactose complexes.**

| Components | Absorbance | Absorption coefficient. ($m^2 mol^{-1}$) |
|---|---|---|
| Free D - lactose 0.1 M | 0.16364 | 0.4091 |
| Ca-lactose complex 0.1 M | 0.07403 | 0.1851 |
| 0.5 M | 0.75344 | 0.3767 |
| 1.0 M | 1.18371 | 0.2959 |

**Table 14 :**

**Analysis of ultrafiltration permeate**

| Component | Cheese factory effluent mg / L | Treatment plant effluent mg / L |
|---|---|---|
| Lactose | 50,000 | 2,000 |
| $K^+$ | 194.00 | 1.353 |
| $Na^+$ | 550.00 | 50.50 |
| $Ca^{2+}$ | 20.00 | 0.011 |
| $Mg^{2+}$ | 90.00 | 0.034 |

**Table 15 :**

**Characterization of effluents and hydrolyzed effluents**

| Samples | COD (ppm) | $BOD_5$ (ppm.) | $P^H$ |
|---|---|---|---|
| **Sample effluents** | | | |
| Cheese factory effluent | 60,600 | 33,300 | 4.9 |
| Treatment plant effluent | 4,415 | 2,300 | 11.9 |
| **Hydrolyzed effluents** | | | |
| Cheese factory effluent | 520 | 300 | 4.5 |
| Treatment plant effluent | 150 | 84 | 4.5 |

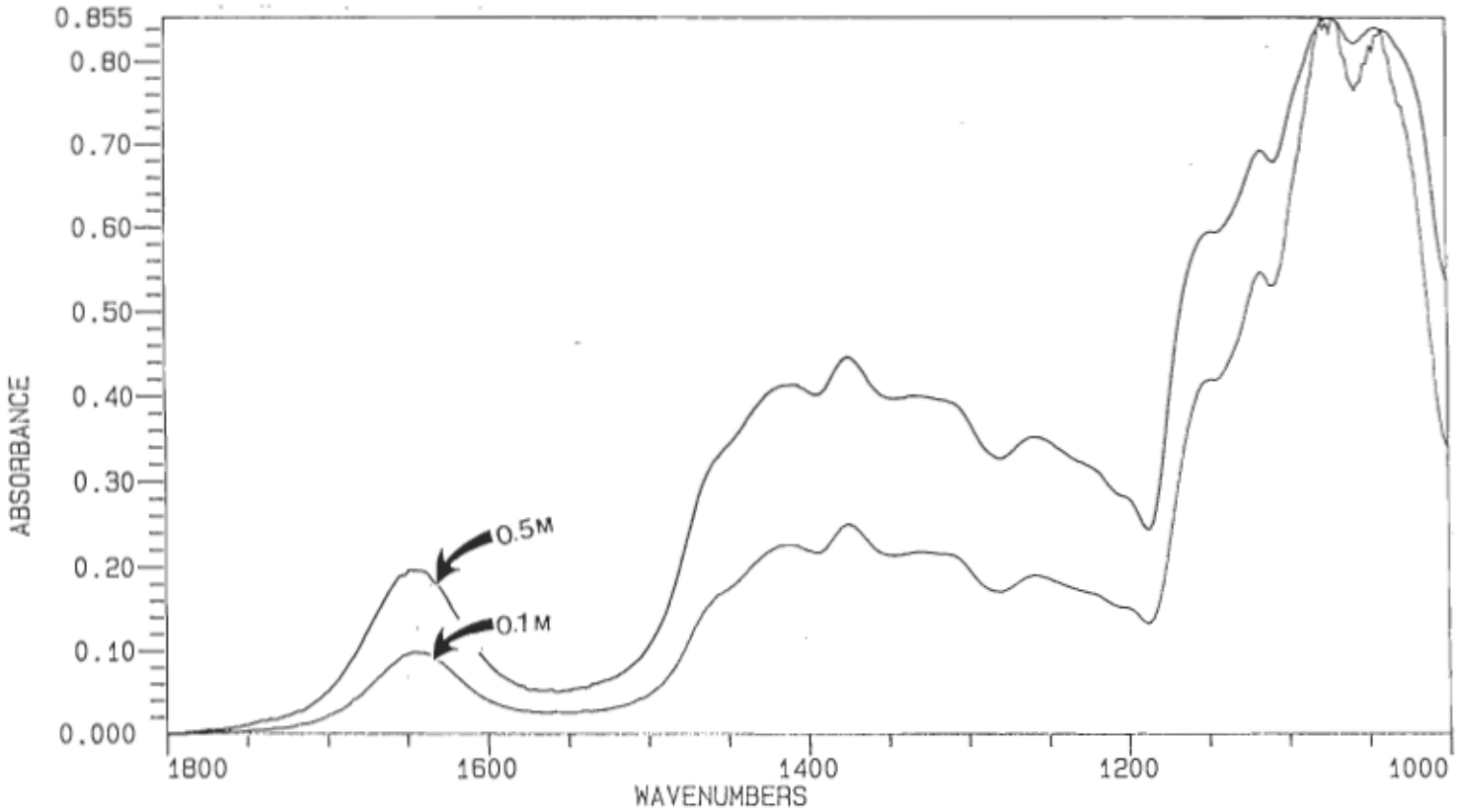



**Figure 13**

Laboratoire de spectroscopie moleculaire

NaCl complex - Lactose
window : CaF2
SCANS: 20 RES: 4.0 TIME: 09/28/95 14:49:32

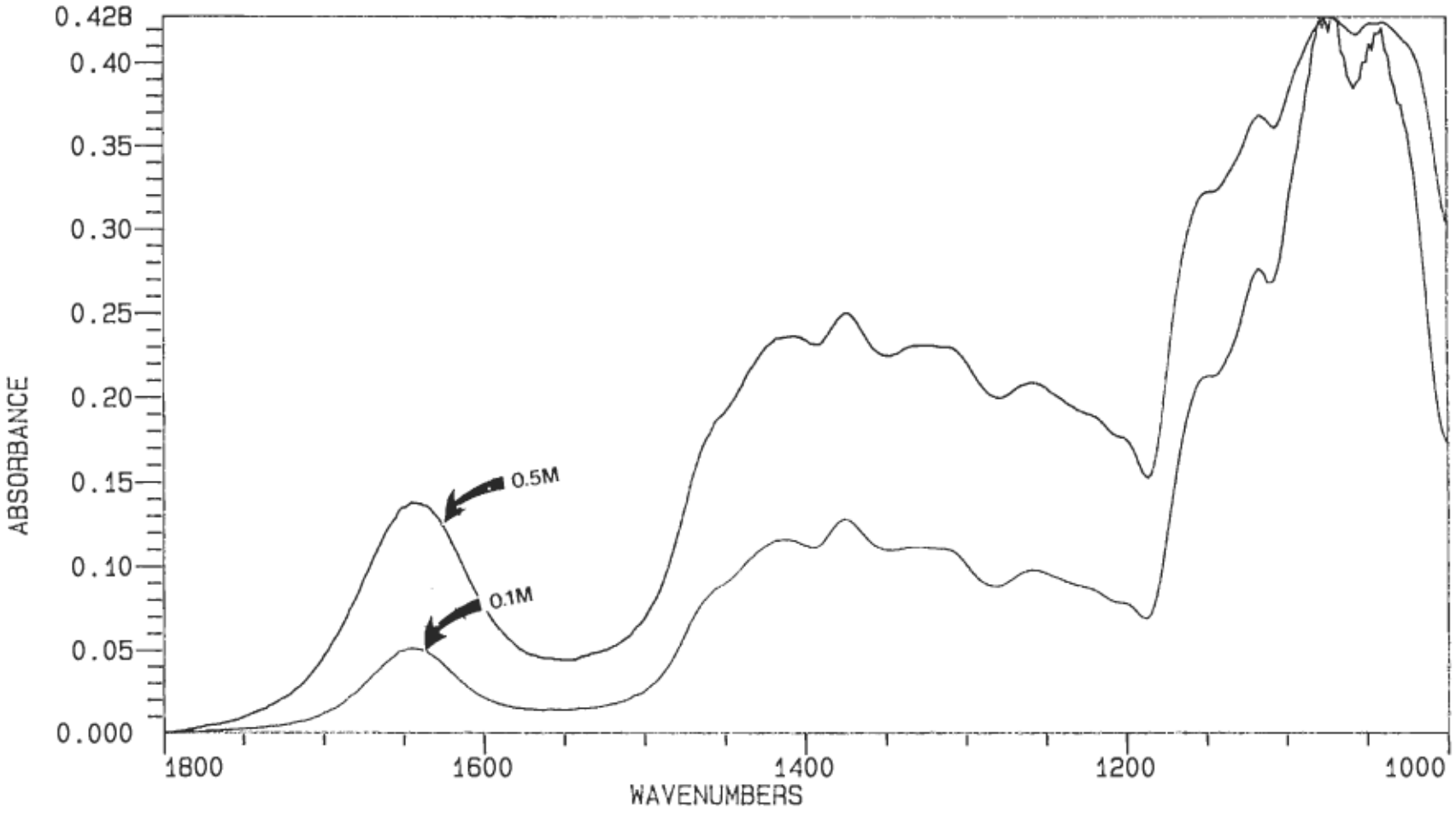



**Figure 14**

Laboratoire de spectroscopie moleculaire

KCl complex
window : CaF2
SCANS: 20 RES: 4.0 TIME: 09/28/95 16:21:55

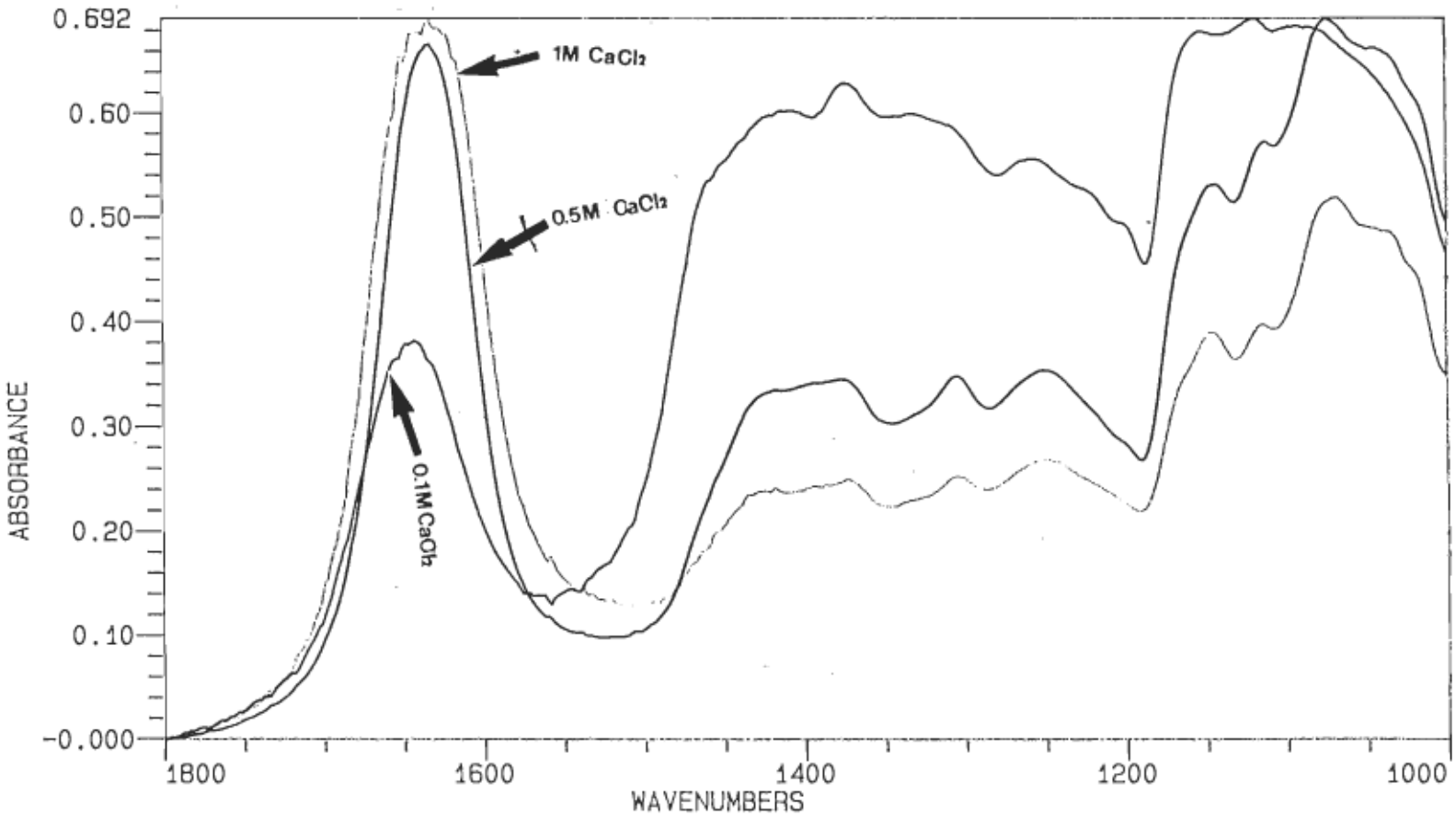



**Figure 15**

Laboratoire de spectroscopie moleculaire

CaCl2 Complex

SCANS: 20 RES: 4.0 TIME: 10/10/95 16:35:39

**Figure 16 : Immobilized Enzyme Hydrolysis of Industrial Cheese Effluent Permeate in Bioreactors at 50 °C.**

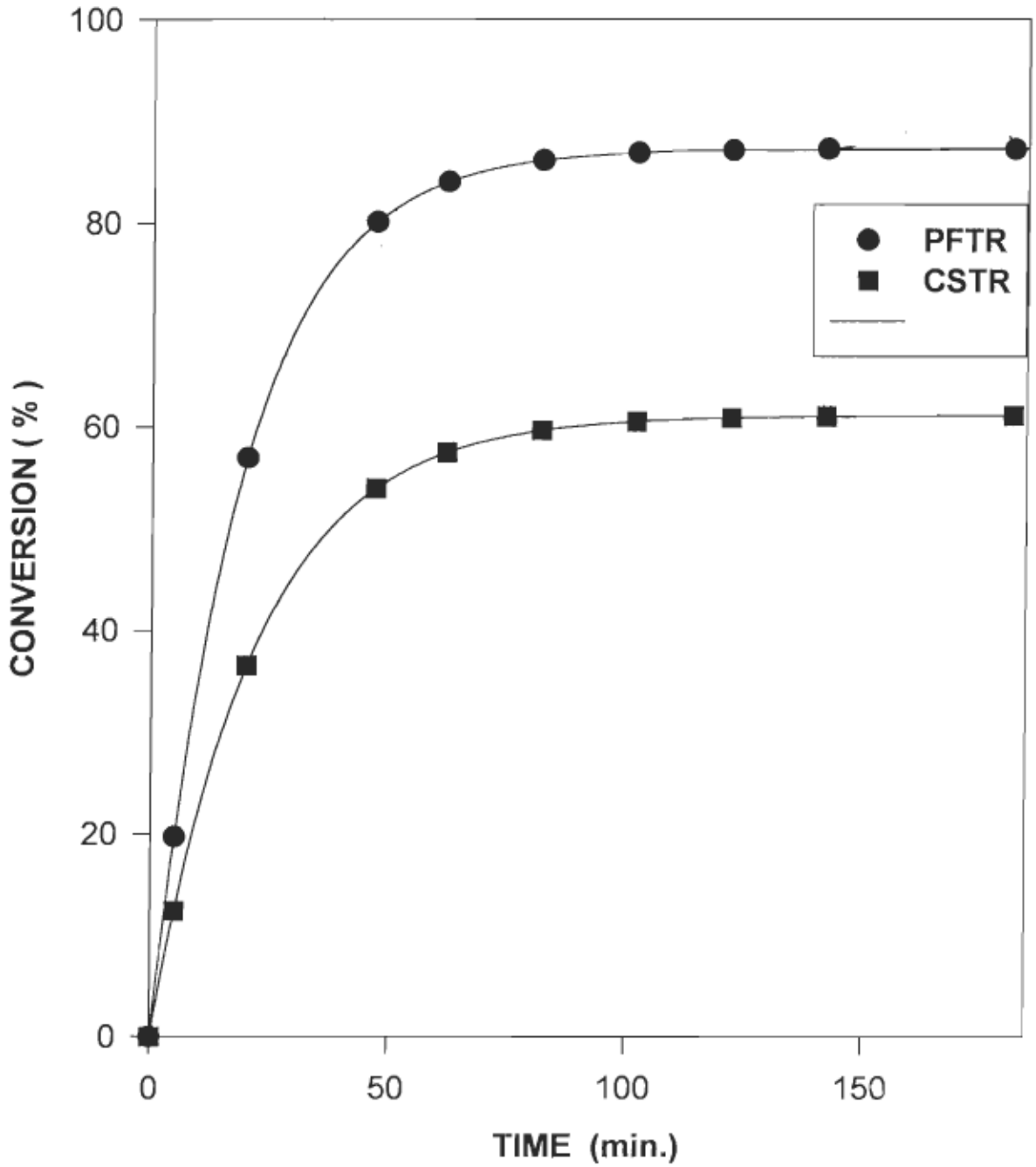
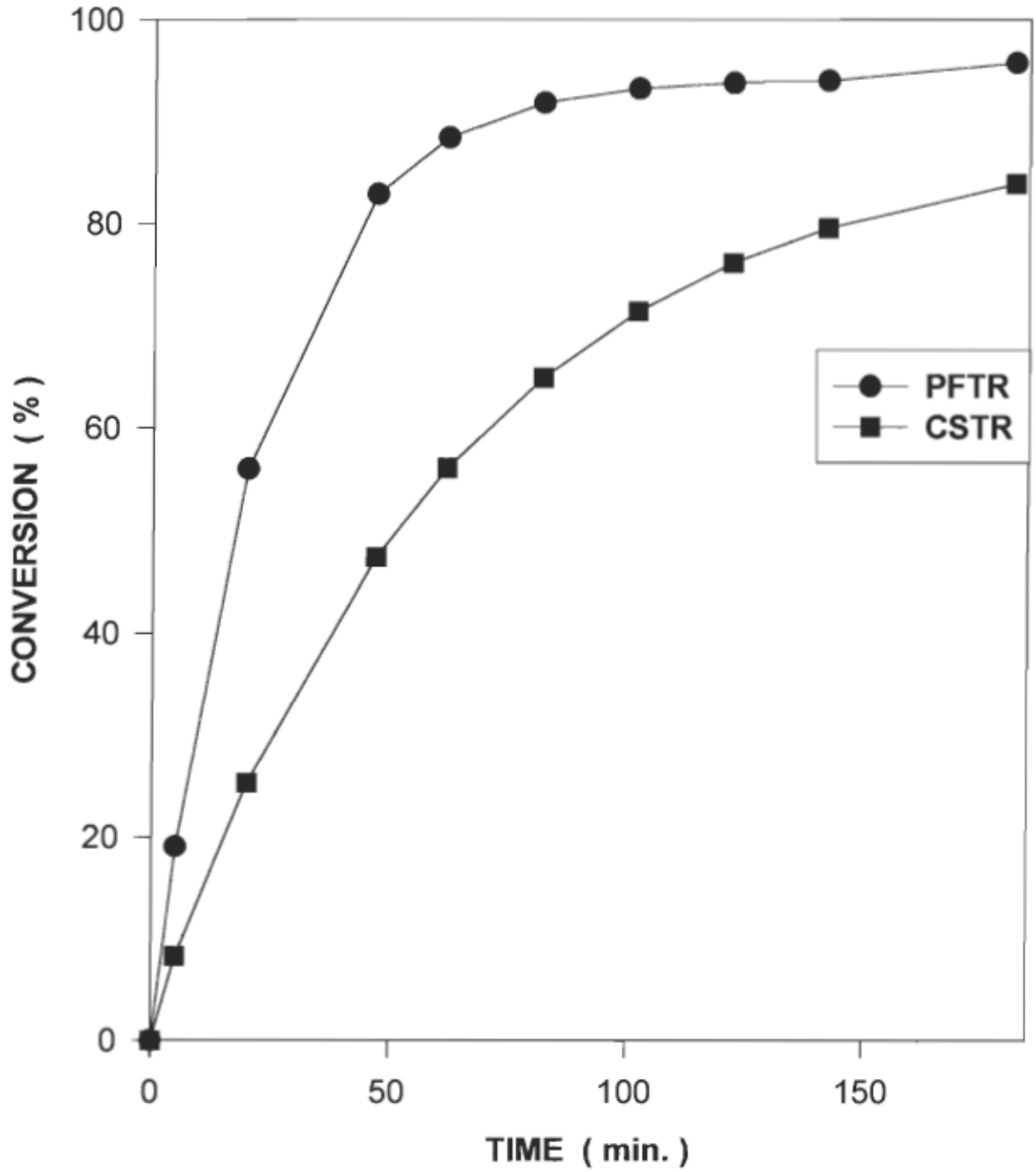


**Figure 17 : Immobilized Enzyme Hydrolysis of Treatment Plant Effluent Permeate in Bioreactors at 50 °C.**



# V. DISCUSSIONS.

## 5.1. Immobilization results

### 5.1.1 Enzyme support

From the experimental results obtained, celite R-640 provided the lowest enzymes loading of 25 mg of enzyme per gm of dry support, but was mechanically unstable in the continuous stirred tank reactor. This was due to its pellet shape. The celite R-640 became fragmented after repeated used. Further more the immobilization efficiency was by far lower than those obtained with GDE biocatalyst carrier as can be seen from table 6. Despite these disadvantages, it seems to be a good biocatalyst carrier in a plug flow reactor.

The GDE biocatalyst carrier had an enzyme loading of 26,3 mg/ g dry (GDE) support. The immobilization efficiency stood at 98,4 %, in addition to its excellent mechanical and thermal stability in both continuous stirred tank reactor and the plug flow reactor. Granular diatomaceous earth support was reused repeatedly for immobilization after regeneration with a simple process involving base-acid wash. This eliminates any disposal problem and provides a potential economic savings as well. These advantages led to the selection of GDE biocatalyst carrier as the principal enzyme support used in this research.

### 5.1.2 Effect of agitating speed on immobilization efficiency.

It can be seen from figure 18, that the agitating speed greatly influenced the immobilization efficiency. An optimum immobilization efficiency greater than 98 percent was obtained at an agitating speed of about 150 rpm for the lactase, *A. oryzae* using GDE as the support [Table 5]. An optimum agitating speed is required to enable the pendant amine reactive moiety left unreacted when the treated biocatalyst was contacted with glutaraldehyde to sufficiently come in contact with the enzyme. The amine group of the enzyme reacts with the unreacted amine reactive moiety forming a covalent bond thereby immobilizing the enzyme.

### 5.1.3 Residual activity.

It can be observed from the results obtained on immobilization that the immobilization efficiency of both enzymes at an agitation speed of 150 rpm was above 98 percent [see table 16]. Experimental data obtained from hydrolyzing ONPG indicated a higher conversion rate for *A. oryzae* as compared to lactozyme. Moreover, scientific literatures[20, 52] indicates that lactase of the lactozyme group produced more oligosaccharides during lactose hydrolysis reaction than fungal lactase such as *Aspergillus oryzae.* The cheese industrial effluent used in this research had a pH 4.9 and immobilized Aspergillus oryzae reacts best at a pH 4.5. Consequently, *Aspergillus oryzae* was chosen as the biocatalyst for this research. *Lactozyme* , with pH 6.5 is most suitable for hydrolyzing sweet whey which has a pH of 6.0.

Figure 18

# EFFECT OF AGITATING SPEED ON IMMOBILIZATION EFFICIENCY.

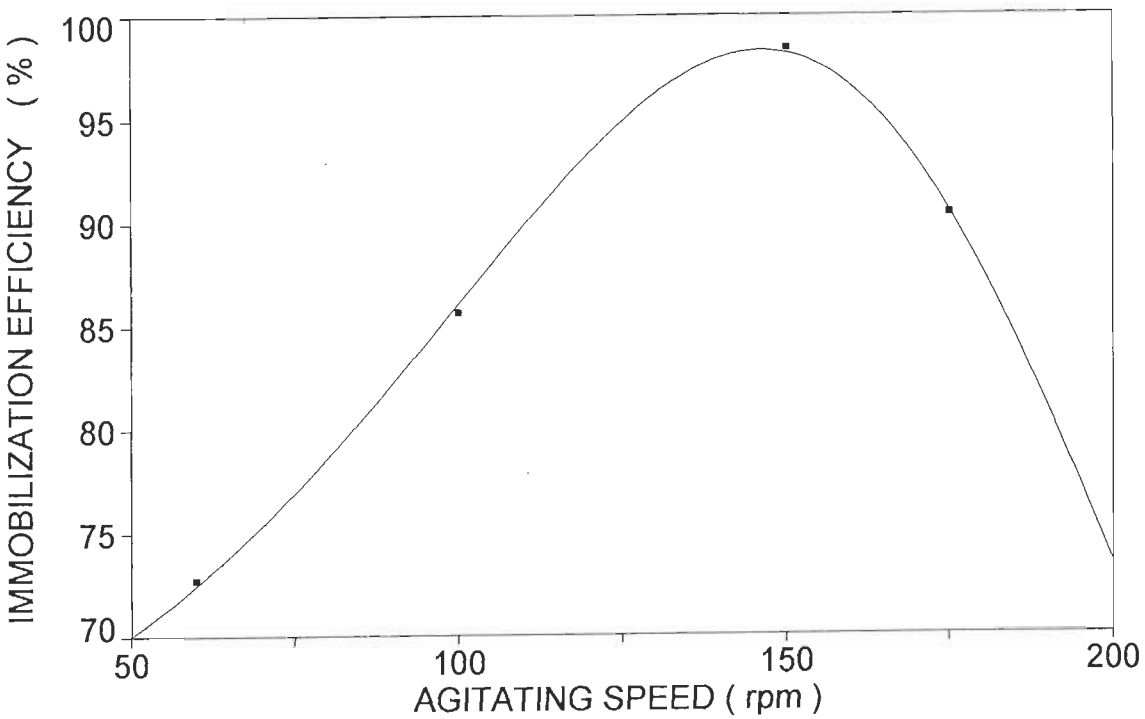



Table 16

**Support : Granular Diatomaceous Earth (GDE).**

| Enzyme | Enzyme Loading mg/g dry wt.support | Initial Activity ONPG units | Immobilization Efficiency ( % ) |
|---|---|---|---|
| *Lactozyme* | 60.0 | 2840 | 99 |
| *Aspergillus oryzae* | 26.3 | 4.66 | 98.4 |

### 5.1.4 Thermal stability of immobilized enzyme.

It can be observed from figure 7 that immobilized Aspergillus oryzae was most stable at a temperature of 50 °C. Similar results were obtained by other researchers [41, 55, 56] using different biocatalyst carriers as well as different immobilization methods.

### 5.1.5 Mechanical stability of immobilized enzyme.

From the experimental data obtained, it can be seen that the optimum mechanical stability was observed at an agitation speed of 120 rpm in the continuous stirred tank reactor [see figure 8 ].

## 5.2. Discussion of kinetic results.

### 5.2.1. Kinetic studies without Cations.

#### 5.2.1.1. Effect of temperature on the kinetic parameters.

The rate of reaction was found to increased with increasing temperature from the experimental results obtained, but became limiting at high temperatures as indicated in figure 19. It is also observed from figure 20 that the maximum conversion increased with increasing temperature, but decreased steadily at temperatures above 55 °C. The values of the Michealis - Menten constant, $K_m$, obtained in this research were smaller than those reported in some papers [56,58] using Michealis - Menten type kinetic with competitive product inhibition.

Figure 19

Effect of Temperature on Maximum Rate of Hydrolysis Reaction.

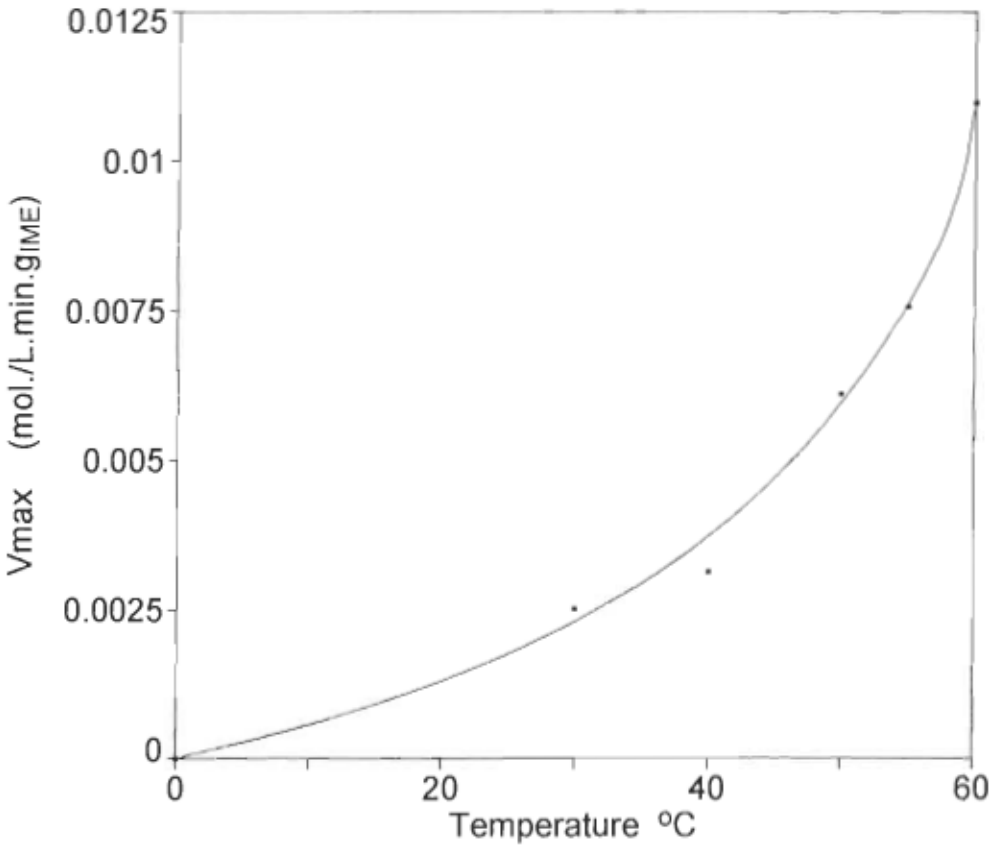
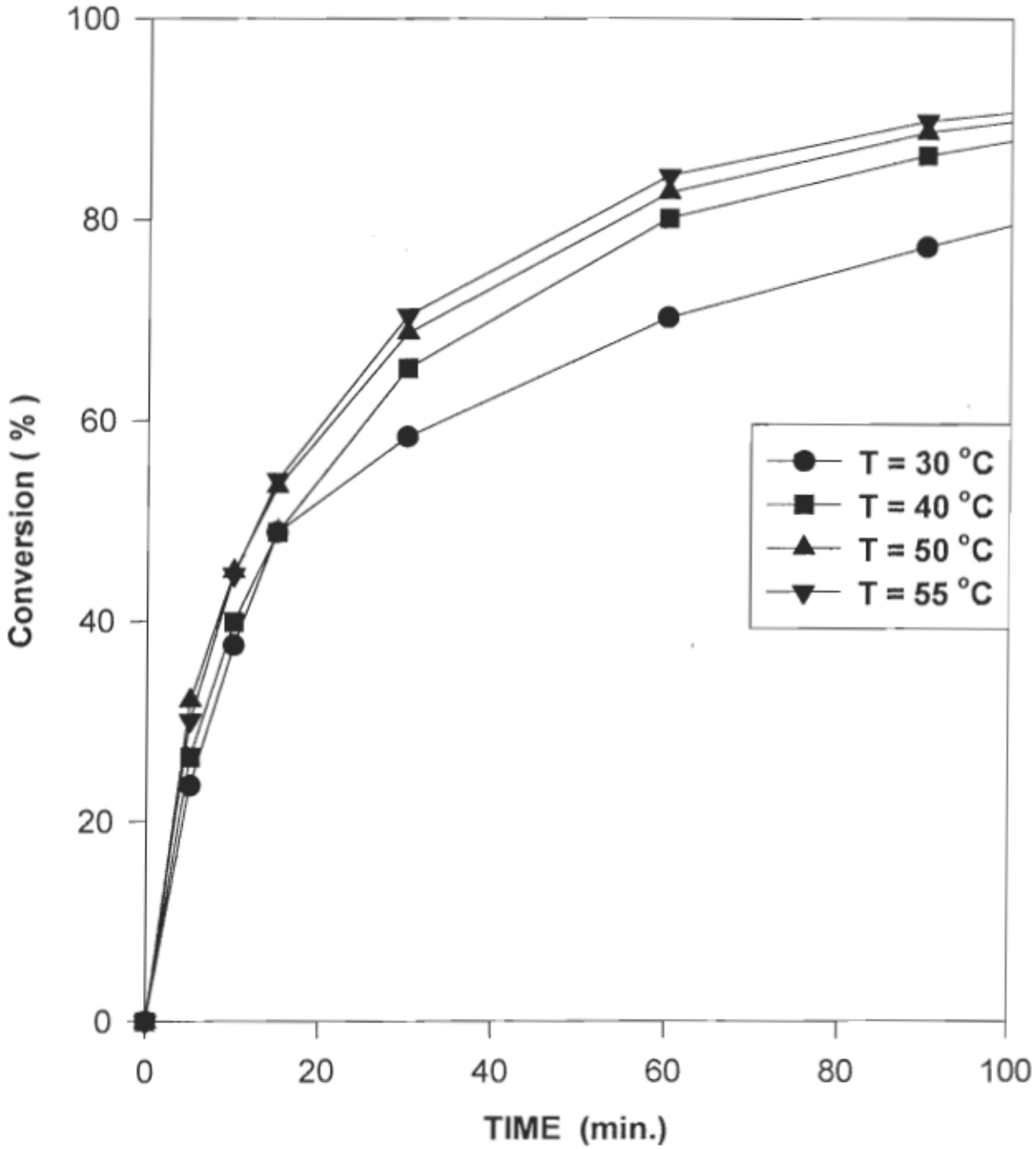


**Figure 20 : Effect of Temperature on Conversion .**



This discrepancy is largely due to fact that the methods used in determining this constant are different but were well within the range of those given in other literature [49, 55]. In this research a non-linear computer regression method was applied to equation 4 used in determining the kinetic parameters, whereas the other researchers used the Lineweaver - Burke method. The kinetic parameters obtained from the Lineweaver-Burke method compared well with those reported by the above paper [58] using the same enzyme under similar conditions. Table 17 summarizes the kinetic parameters obtained in this research using Lineweaver-Burke method.

Published values of $K_m$ vary between 18mmol/L[59] and 160 mmol/L[60] for immobilized *Aspergillus oryzae* under similar operating conditions. The Michealis-Menten kinetic with competitive product inhibition represented by equation 4 was used as the kinetic model because *Aspergillus oryzae* is severely inhibited by the hydrolysis product, galactose.

### 5.2.1.2. Effect of initial lactose concentration on the kinetic parameters.

From the kinetic parameters obtained and summarized in table 8, it can be noticed that lactose concentration increases with the maximum rate of reaction. However, an extrapolation of lactose concentration as a function of $V_{max.}$( maximum rate of reaction) indicated a limit at high substrate concentration as shown in figure 21.

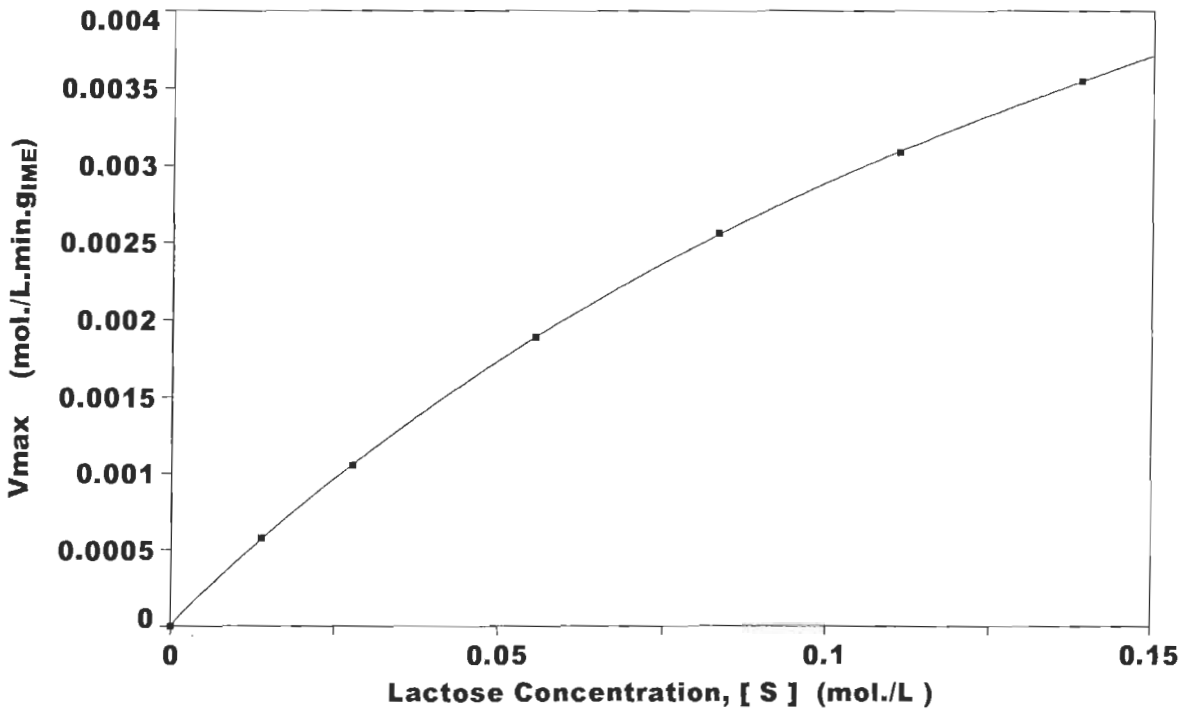
Table 17:

Kinetic parameters from Lineweaver-Burke method.

| TEMPERATURE ° C | Vmax. mmol./L.min.$g_{IME}$ | Km app. mmol./ L |
|---|---|---|
| 30 | 3.98 | 34.15 |
| 40 | 4.17 | 56.30 |
| 50 | 5.14 | 58.30 |
| 55 | 7.23 | 66.46 |
| 60 | 2.12 | 40.56 |

**Figure 21**

**Effect of Initial Lactose Concentration on Maximum Rate of Hydrolysis Reaction.**



It was also noted from the degree of hydrolysis curve that maximum conversion was affected by lactose concentration. It can be observed from figure 22 that conversion decreased as lactose concentration increased, indicating that lactose and galactose may be involved in oligosaccharides formation reactions. Fungal lactase have long been reported by several researchers[20, 47,57] to transform transgalatosyl reactions to oligosaccharides . This explains why galactose was lower than glucose in most of the reaction medium. Even though it has been demonstrated that oligosaccharides are formed during the hydrolysis of lactose, it was neglected in the proposed rate of reaction model. This is because one study proved that oligosaccharides formation could be neglected in the modeling of lactose hydrolysis reaction when initial lactose concentration is low and the reaction temperature is about 50 °C [21].

## 5.2.2. Kinetic studies in the presence of cations.

### 5.2.2.1 Effect of single cations on the kinetic parameters.

The rate of lactose hydrolysis is greatly influenced by the presence of cations as can be seen from figure 23. On comparing the values of the kinetic constants obtained in the presence of cations to those in the absence of cations, as represented in table 9, it was observed that the presence of divalent cations played a significant role on the activity of the enzyme. Interestingly, the kinetic parameters were higher for $Ca^{++}$ and $Mg^{++}$ as compared to those of $K^{+}$ and $Na^{+}$ as

**Figure 22 : Effect of Initial Lactose Concentration on Conversion at 50 °C.**

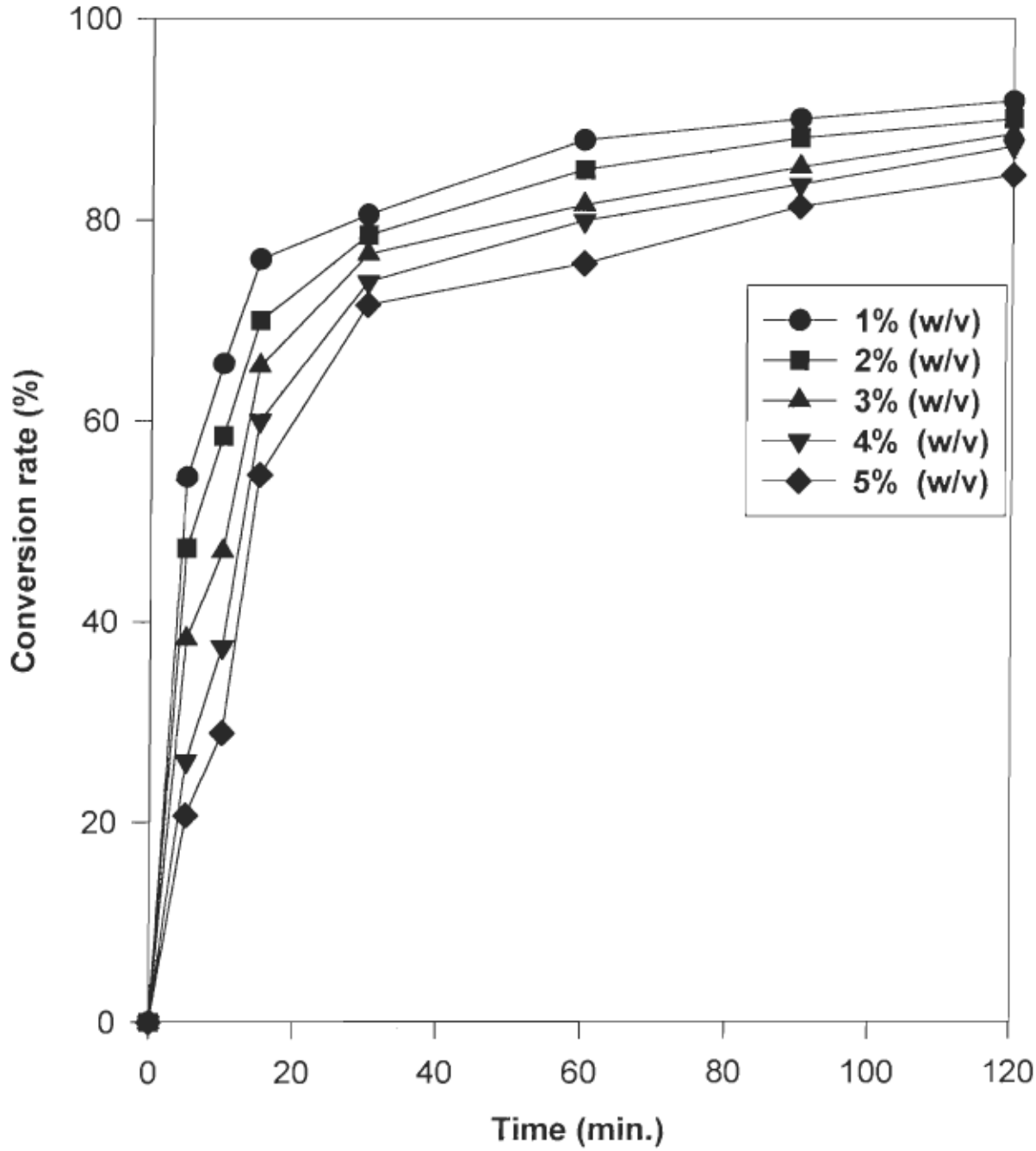
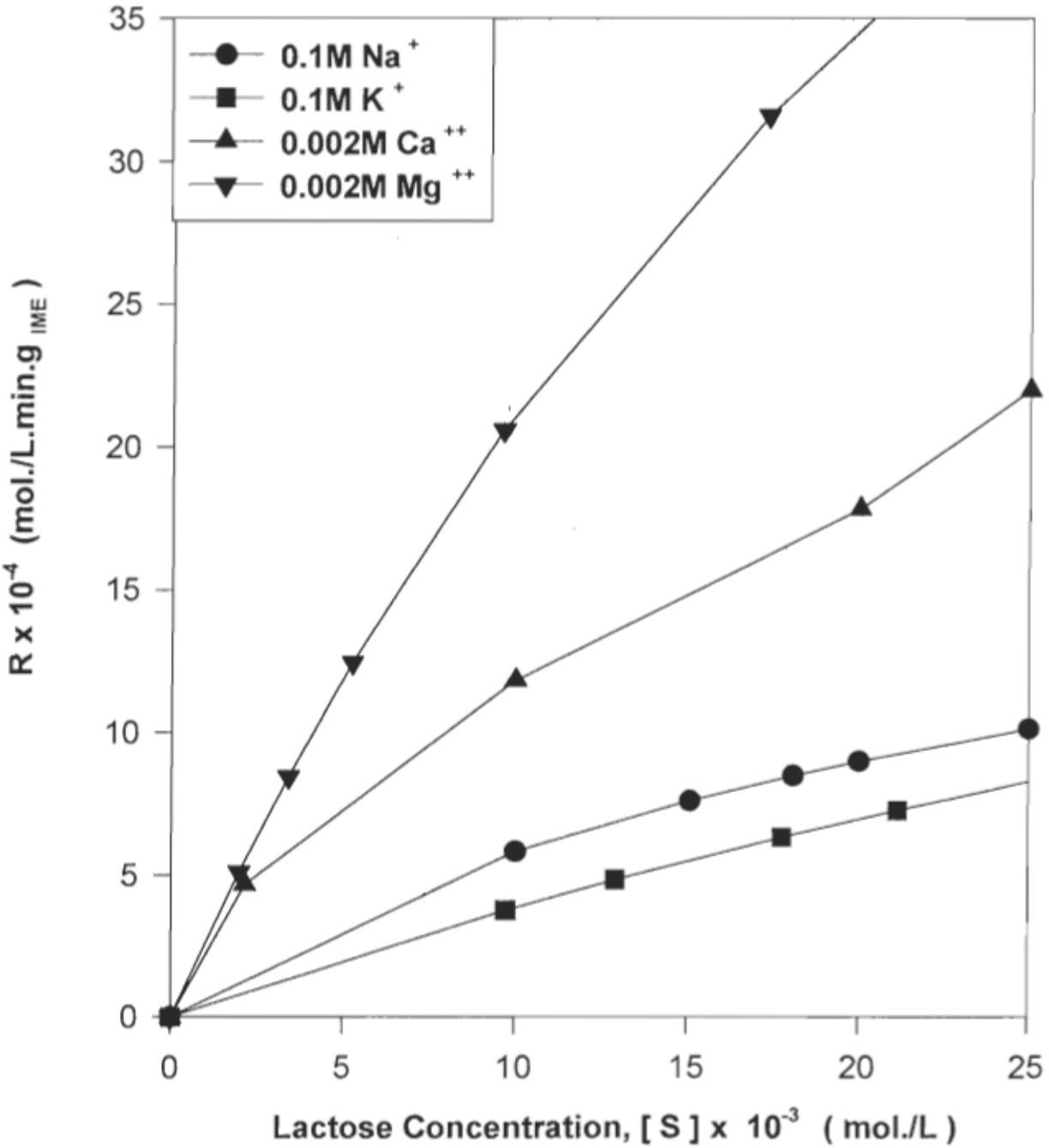


**Figure 23 : Effect of Single Cation on the rate of Lactose hydrolysis reaction.**



well as those obtained in the absence of cations. Surprisingly, the kinetic parameters obtained in the presence of monovalent cations were slightly lower than those obtained in the absence cations. As expected, higher maximum conversion rates were obtained in the presence of divalent cations than in the presence of monovalent cations as shown in figure 12.

### 5.2.2.2. The effect of combined cations on the kinetic parameters.

When the three most abundant cations found in cheese industrial effluent were combined in the concentration ranges mentioned in the experimental section, complex results were obtained. Keeping the $K^+$ and $Na^+$ concentration constant at 75mM : 75mM and varying $Ca^{++}$ concentration from 0.5 mM - 2 mM, the results represented in figure 24 were obtained. It indicated that the rate of lactose hydrolysis reaction increased with increasing $Ca^{++}$ concentration as shown in table 10. However, a graph of the effect of increasing $Ca^{++}$ concentration on maximum rate of reaction obtained in this research indicates that this increased in the maximum reaction rate is limited as can be seen in figure 25. An extrapolation of this curve indicates that Vmax is limited.

Similarly, while keeping $K^+$ and $Ca^{++}$ concentration constant at 50mM : 0.5mM respectively , and varying $Na^+$ concentration between 50mM - 100 mM, the maximum reaction rate was noted to be increasing. This increased was limited at

high $Na^+$ concentration. Figure 26 represent the variation of $Na^+$ concentration with reaction rate, while figure 27 shows the effect of $Na^+$ concentration on Vmax. An optimum $Na^+$ concentration can be obtained from figure 27 by extrapolation.

It was observed from figure 28 that the concentration of $K^+$ increased with increasing reaction rates but at concentration above 0.08 M, it decreased steadily as can be seen in figure 29.

**Figure 24 : Effect of [Ca $^{++}$] on the rate of Lactose Hydrolysis reaction.**

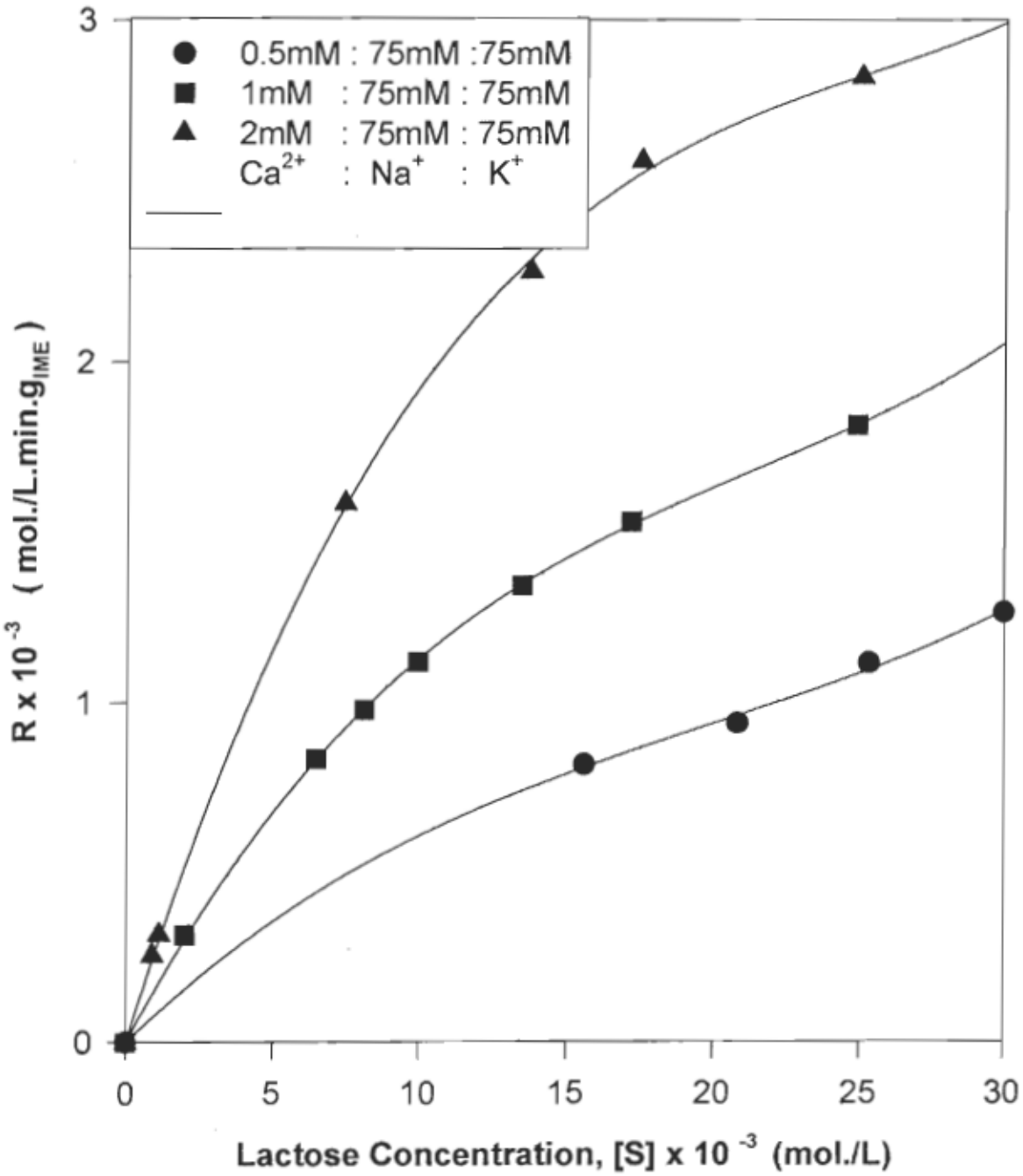


**Figure 25**

**Effect of $Ca^{++}$ on Maximum Rate of Hydrolysis Reaction in the Presence Cations.**

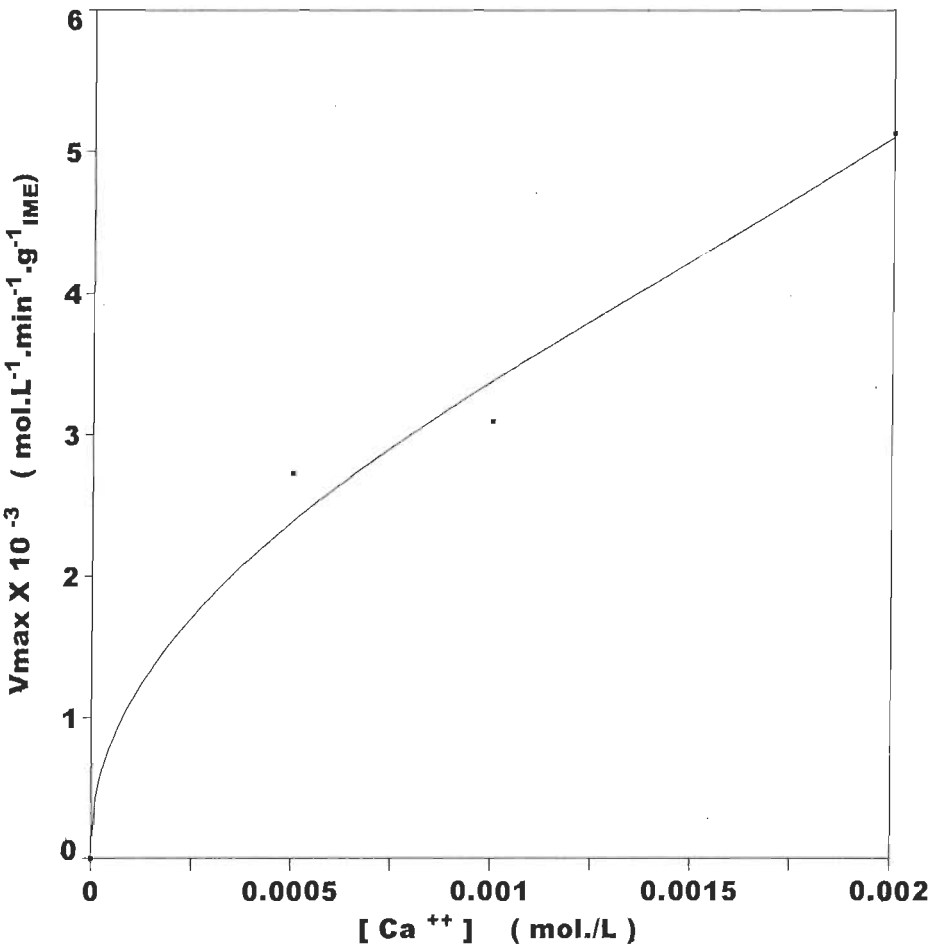


**Figure 26 : Effect of $Na^+$ on Rate of Lactose Hydrolysis Reaction at 50 °C.**

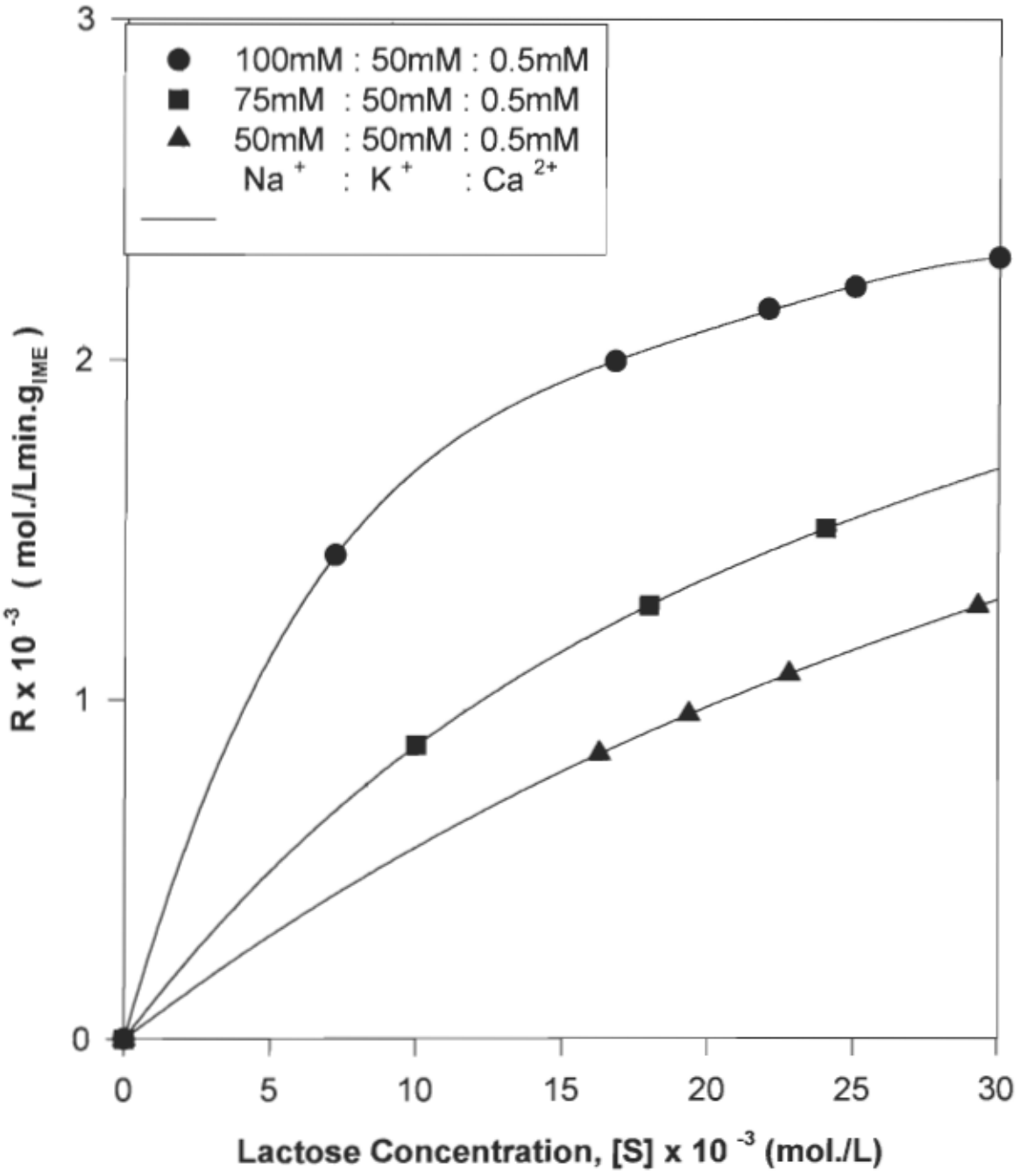
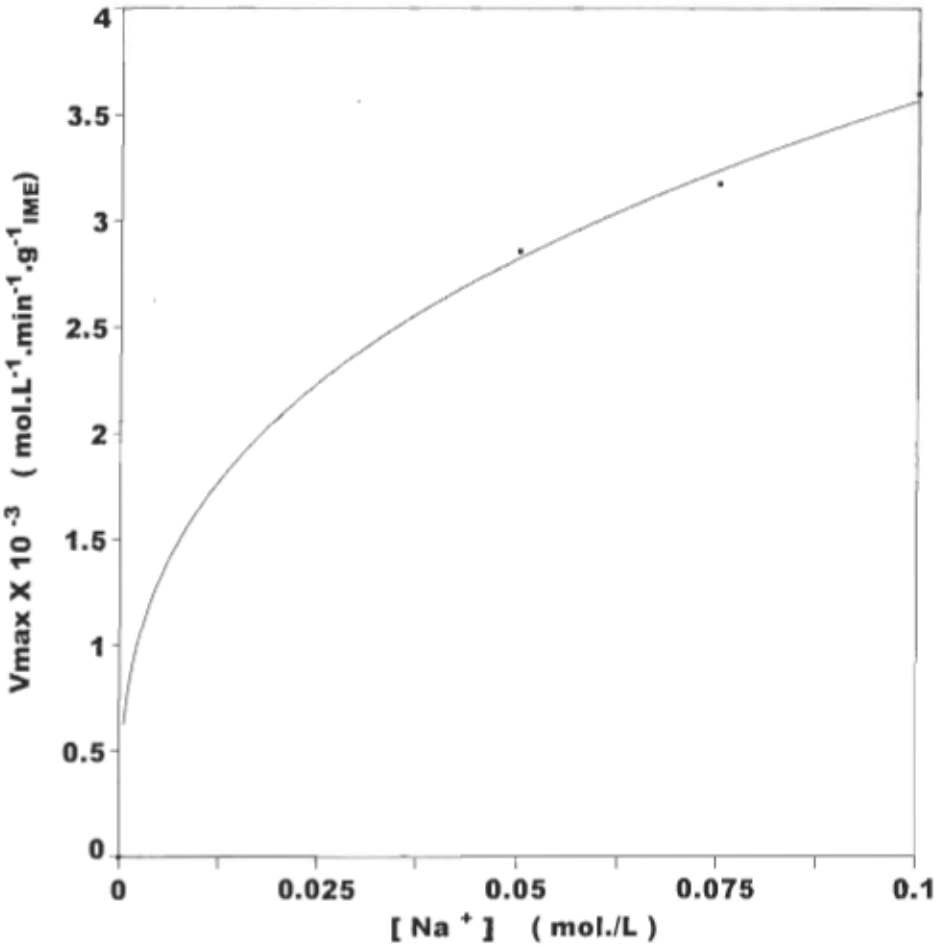


Figure 27

**Effect of $Na^+$ on Maximum Rate of Hydrolysis Reaction in the Presence of Other Cations.**



## Figure 28 : Effect of $K^+$ on the rate of Lactose Hydrolysis reaction at 50 °C.

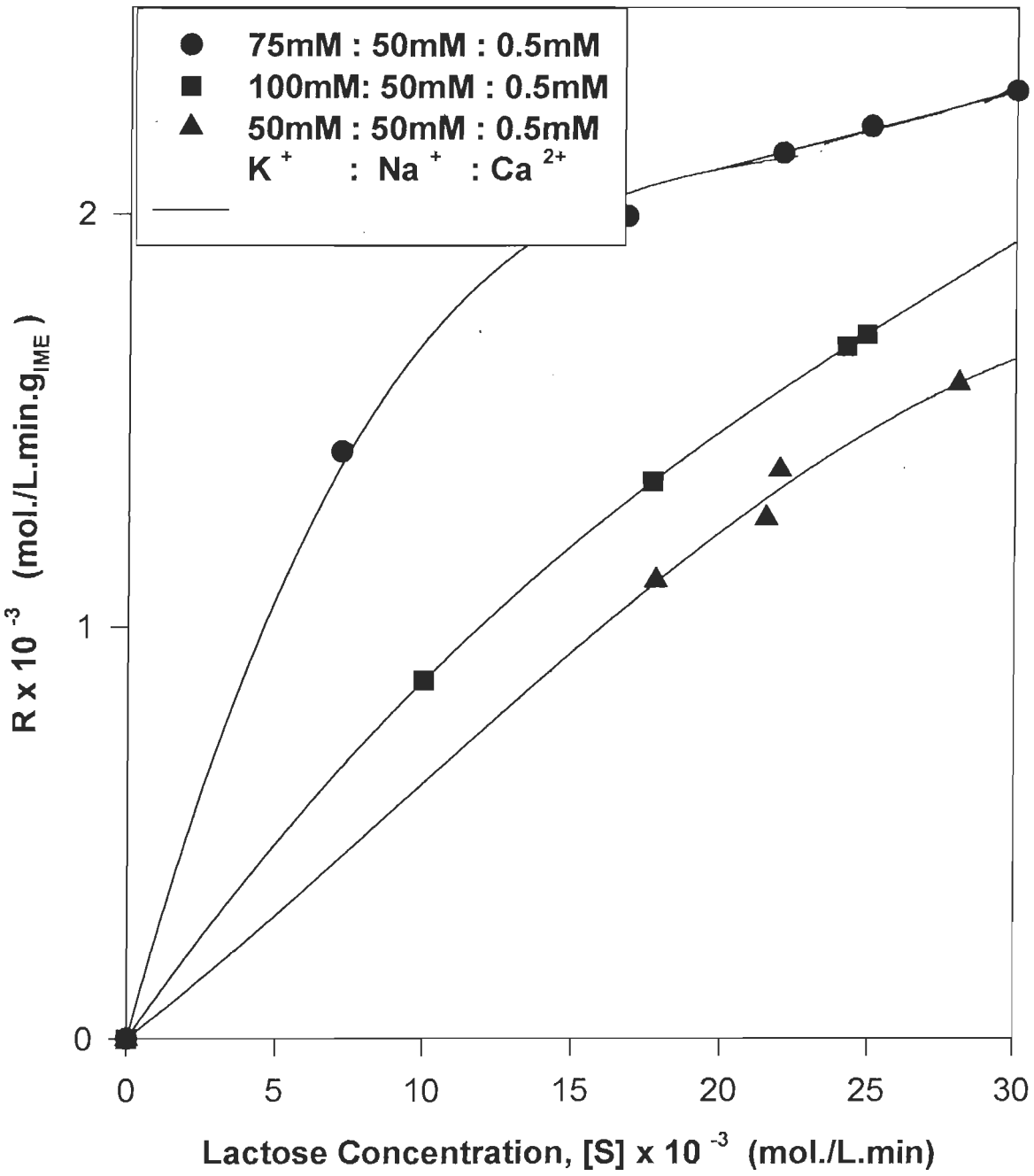
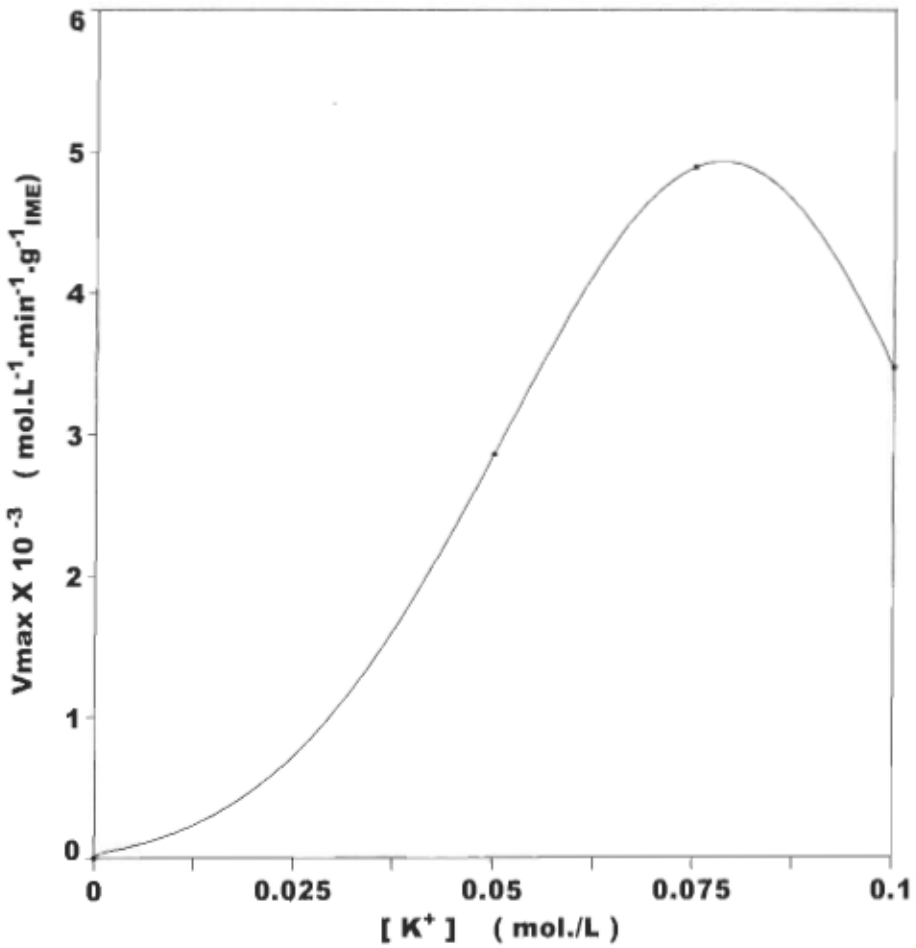


**Figure 29**

## Effect of $K^+$ on Maximum Rate of Hydrolysis Reaction in the Presence of Other Cations.



Finally, the effect of increasing the concentrations of combined cations on the kinetic parameters is given in table 18. From this table, the highest maximum rate of reaction of 6.63 $mmol.L^{-1}.min^{-1}.g^{-1}_{IME}$ and a $K_{m.app.}$ value of 72.69 mmol./L was observed for a combination of three cations at a concentration of Na : K : Ca being 100 : 100 : 1 respectively.

Experimental data suggest that the effects and interactions observed from the combined ($Na^+$, $K^+$, $Ca^{2+}$ ) cations were quite complicated and differ significantly from individual cations. The highest maximum activity (Vmax = 9.68 $mmol.L^{-1}.min^{-1}.g^{-1}_{IME}$ ) was given by $Mg^{++}$ whereas, the highest activity from the combined cations, 6.63 $mmol.L^{-1}.min^{-1}.g^{-1}_{IME}$ was barely above the Vmax (6.11 $mmol.L^{-1}.min^{-1}.g^{-1}_{IME}$) obtained in the absence of cations. The $K_M$ values obtained in the presence cations were smaller, indicating a greater affinity of the enzyme for lactose[61]. Consequently, the binding of cations to the enzyme - lactose complex is much stronger.

## 5.3. Tentative explanation of the cation - lactose complex mechanism.

Although sugars interaction with alkali and alkaline earth metal ions are well known [62, 63], there has been no report on lactose - metal ion interaction in literature so far. In this study, infrared spectra of lactose - cations complexes and free lactose were recorded in the region of 1800-1000 $cm^{-1}$ and the results of the

spectral analysis is described. In this region only one group of absorption band was observed at approximately 1650 $cm^{-1}$ . This group of strong and broad absorption band can be assigned to the OH groups of the water molecule. The band at 1650 $cm^{-1}$ in the spectrum of free sugar appeared as a sharp absorption band, but it showed considerable intensity changes upon lactose metalation with 0.1M of hydrated metal halide ions. Further variation of KCl ions concentrations showed no significant changes in the absorption band whereas NaCl ions concentration showed no significant changes in the absorption band only after 0.5 M. However, the intensity change observed at 0.5M for monovalent metal ions indicated a greater absorption for $Na^+$ as compared to $K^+$ complexes [figure 30]. This dissimilarity is due to the smaller ionic raduis of $Na^+$ with respect to $K^+$. Spectroscopic evidence indicated that K(D-lactose)Cl. $nH_2O$ and Na(D-lactose)Cl. $nH_2O$ were isomorphous in term of coordinate ion geometries and had similar binding-sites [64]. The interaction between the potassium or sodium atom and the oxygen molecule in D - lactose ring is largely electrostatic [65] and it is mainly through cation hydration shell.

The band at 1650 $cm^{-1}$ in the spectrum of Ca(D-lactose)$Cl_2$. $nH_2O$ complex showed distinct intensity changes and shifted towards a lower absorption frequency of 1637 $cm^{-1}$ as the $Ca^{++}$ concentration was increased from 0.1M to 0.5M, then to 1M $CaCl_2$ [figure 15]. It is worth noting that the skeletal vibrations,

C-C and C-O-C of the lactose ring around 1500 - 1000 $cm^{-1}$ showed changes upon metalation [Figure 15]. The spectral changes observed for these vibrations are due the involvement of the lactose donor atoms in the metal-ligand bonding, as well as the rearrangement of the strong hydrogen bonding network of the free lactose on the complex formation, thereby causing a large perturbation to the ring system where the vibrations are mostly localized and finally bringing a distortion to the ring system. The lactose OH and C-O groups, as well as the water molecules are involved in the cation - lactose bonding.

Even though X-ray and NMR spectroscopy have often been used to characterized the nature of metal - carbohydrate interaction [68, 69 70], FT-IR study could lead to a reliable correlation between the missing structural information and the spectroscopic properties for sugars. Metalation of the sugar moiety can be a useful probe in order to develop our understanding about lactose - cations interaction and vibrational frequencies and to relate them to the kinetics of enzymatic hydrolysis of cheese industrial effluent and other sugar containing substrates. The strong interaction between calcium ions and the lactose molecule as compared to sodium or potassium ions observed in this spectroscopic study indicates that the lactose - enzyme complex has a greater affinity for divalent cations ($Ca^{2+}$ , $Mg^{2+}$ ) than for monovalent cations ($Na^{+}$, $K^{+}$ ). Consequently, the binding of divalent cations to the enzyme - lactose complex is much stronger and

resulting to a higher maximum rate of reaction as well as a higher degree of hydrolysis as compared to monovalent cations or the absence of cations i.e. demineralized cheese effluent. This can be confirmed by the fact that the $K_{m.app.}$ values obtained in this research [table 9] were much smaller for divalent cations as compared to monovalent cations. The smaller the $K_{m.app.}$ value, the stronger the affinity of enzyme for the substrate.

## 5.4. Hydrolysis of cheese industrial effluent in bioreactor .

From figure 16 and figure 17, it can be seen that the rate of conversion of lactose into glucose and galactose is higher in the plug flow tubular reactor (PFTR) than in the continuous stirred tank reactor (CSTR). This confirmed earlier studies by Ander Axelsson and Guido Zacchi [55] in 1990. The percentage conversion of substrate to product decreased initially with time before settling to a steady conversion rate. Similar observation had been reported in literature [70, 71] stating that deactivation of enzyme was more important initially and slow down after a few days. This decreased was due to enzyme deactivation.

**Table 18:**

**Effect of increasing combined cation on kinetic parameters.**

| Combined[$Na^+$]: [$K^+$] : [$Ca^{++}$] Concentration (mmol./L) | Vmax. mmol./L.min.$g_{IME}$ | Km app. mmol./ L |
|---|---|---|
| 50 : 50 : 0.5 | 2.85 | 7.222 |
| 50 : 75 : 0.5 | 4.89 | 46.19 |
| 50 : 100 : 0.5 | 3.47 | 40.83 |
| 75 : 50 : 0.5 | 2.72 | 37.46 |
| 75 : 75 : 0.5 | 6.63 | 72.69 |
| 75 : 100 : 0.5 | 3.60 | 53.40 |
| 100 : 50 : 0.5 | 6.30 | 67.03 |
| 100 : 75 : 0.5 | 3.53 | 44.02 |
| 100 : 100 : 0.5 | 3.82 | 43.07 |
| 50 : 50 : 1 | 6.31 | 75.36 |
| 50 : 100 : 1 | 2.77 | 21.70 |
| 75 : 75 : 1 | 3.09 | 17.55 |
| 75 : 100 : 1 | 2.40 | 24.04 |
| 100 : 50 : 1 | 2.80 | 60.25 |
| 75 : 75 : 2 | 5.13 | 17.20 |
| 75 : 100 : 2 | 1.15 | 1.210 |
| 100 : 50 : 2 | 2.54 | 6.722 |

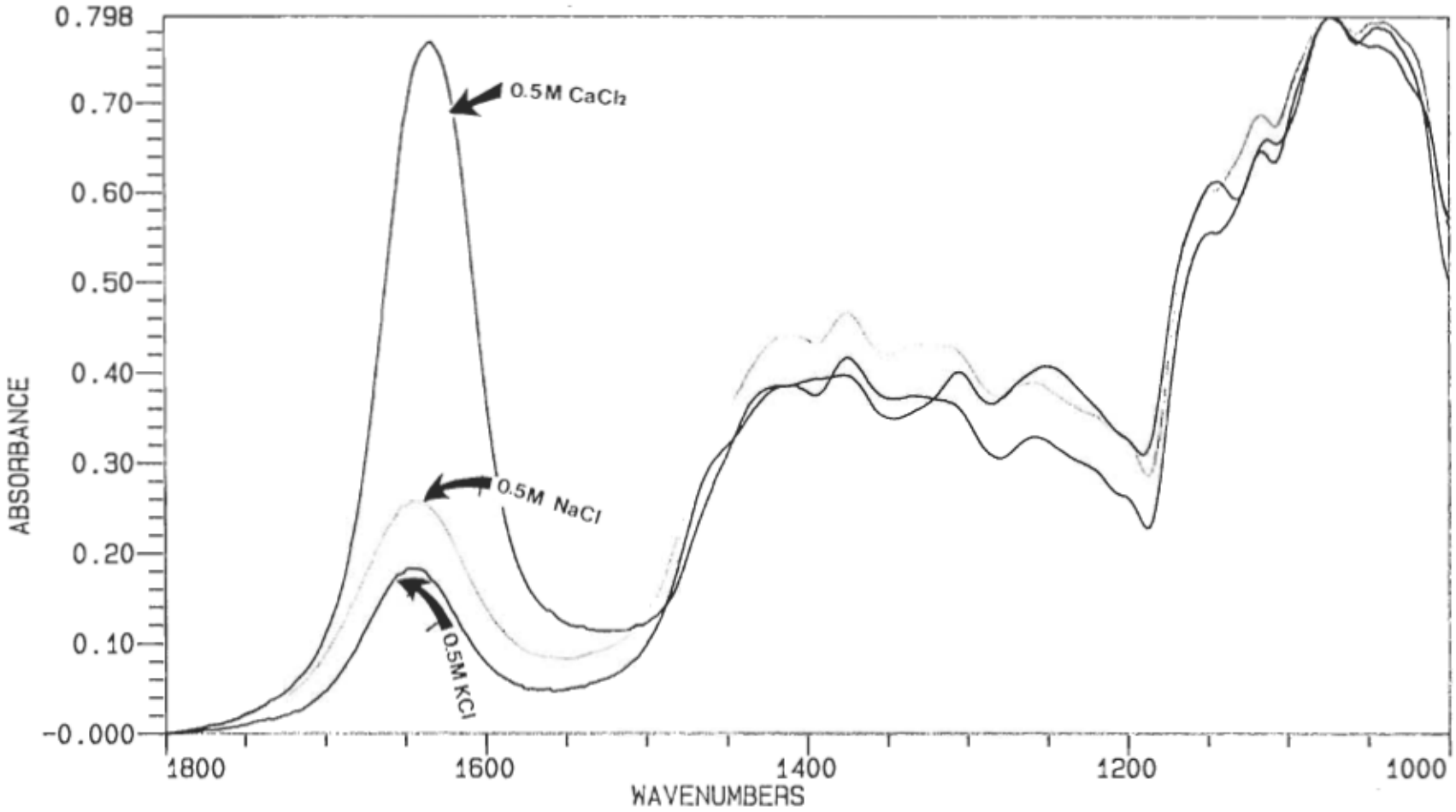



**Figure 30**

Laboratoire de spectroscopie moleculaire

0,5M Ca complex - [S]
window : CaF2
SCANS: 20 RES: 4.0 TIME: 09/29/95 16:11:56

There are three possibilities by which a constant production and conversion rate can be maintained by :

- Raising the temperature to compensate for activity loss [47, 55, 71] .
- Performing reaction at isothermal conditions using multiple-reactors system[51, 55]
- Reducing flow of substrate and by so doing the residence time is increased [21] .

## 5.5. Immobilized enzyme deactivation and half - life estimation.

In order to determined the deactivation of an immobilized enzyme, the reactor performance at constant temperature and flow rate was monitored for a long operating time. Experimental data on lactose conversion were collected and used in evaluating the deactivation constant and then , the half-life. In order to evaluate the deactivation kinetics, the experimental data obtained from the plug flow tubular reactor was used. The proposed equation 21 for an ideal plug flow reactor was used. This equation could be rearranged to give the expression :

$$\frac{W}{F} Eo * \exp(-k_d * t) = -A(1 - X) + BX \qquad (24)$$

$$\Rightarrow \ln\left[\frac{WEo}{F}\right] - K_d * t = \ln\left[-A\ln(1 - X) + BX\right] \qquad (25)$$

On performing the linear plot of Ln [-Aln(1-X) + B*X] as a function of time, t , a straight line graph is obtained as shown in figure 31 with the slope equal to - $K_d$; the deactivation constant.

The observed deactivation constant was best described by a first order reaction mechanism, reported to fit the thermal deactivation of lactase and it was in accordance to earlier reports by some researchers under similar conditions[21, 54] The residual activity or the exponential decay of an immobilized enzyme is represented by equation 20. By substituting the deactivation constant obtained in equation 25 into equation 20 , the half - life of the immobilized enzyme can be determined.

The half-life, $t_{1/2}$, is described as the time the initial activity of the IME drop by 50 percent; i.e. when $E = \frac{Eo}{2}$, and can be calculated by substituting into equation 20 to obtain

$$\frac{1}{2} = e - K_d * t_{\frac{1}{2}} \tag{26}$$

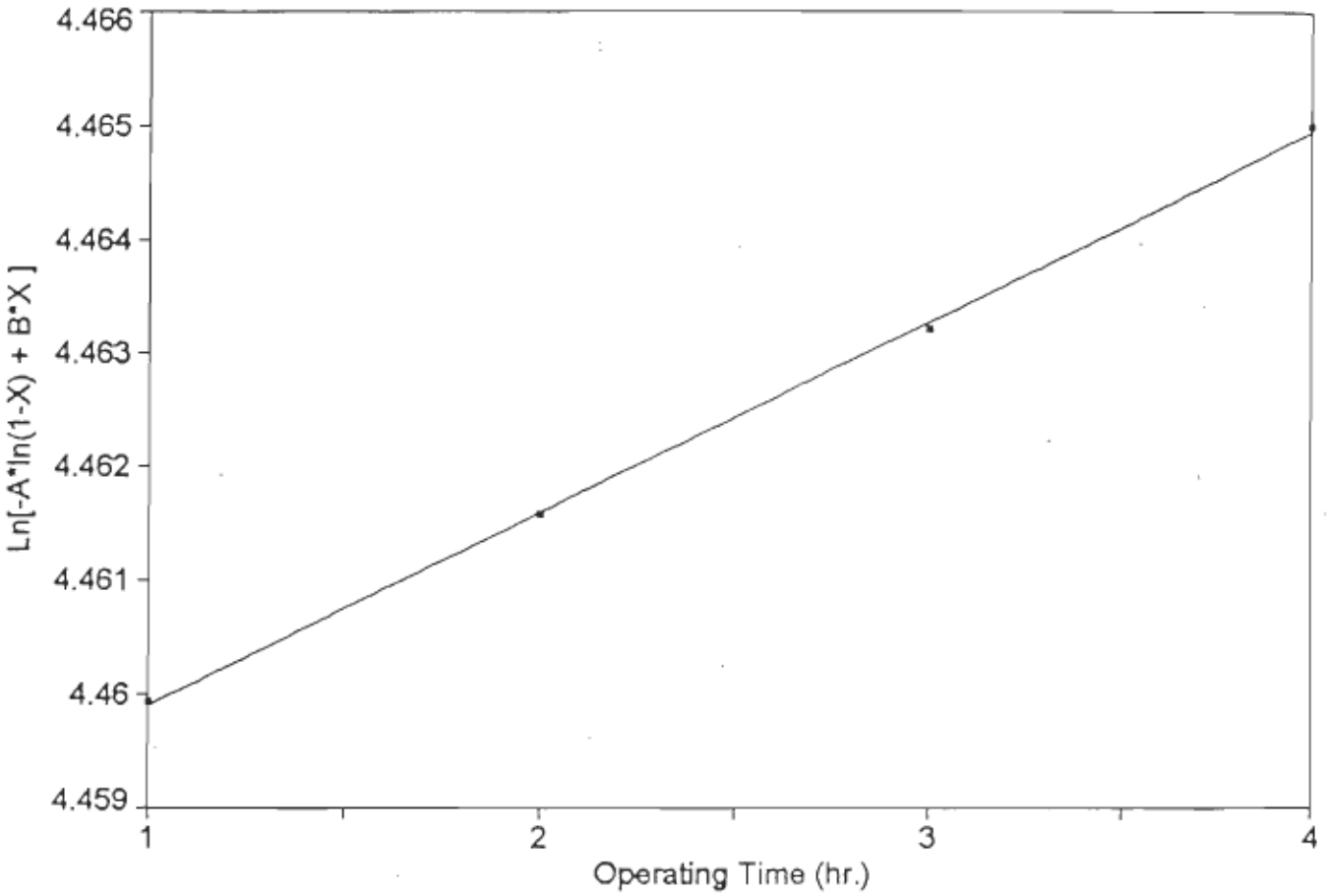
$$\Rightarrow t_{\frac{1}{2}} = \frac{\ln 2}{K_d} \tag{27}$$

The estimated value for the deactivation constant, $K_d$, was 17 days. Under the actual operating conditions, one - half of the initial activity was lost after 17 days. For comparison, Friends and Şhahani [60] using the enzyme, A. oryzae and at same temperature, 50 °C observed a half-life (first order) of 13 days. The deactivation of enzymes are mainly influenced by the operating temperature, pH, nature of the feed, and microbial contamination. From the batch process it was observed that at lower temperatures , example at 30 °C, more galactose was

present in the analyzed products than glucose. At this temperature the half-life was reported to be longer than at a temperature of 50 °C by S.T. Yang [21] and he reported that galactose in fact offered a protective effect on the activity of enzyme while the effect of glucose was the contrary. However, at lower temperatures more oligosaccharides (undesirable products) are formed thereby lowering the degree of conversion of lactose to glucose and galactose.

Figure 31

Determination of Deactivation Constant, Kd from Plug Flow Reactor Performance.



# VI. ECONOMIC ANALYSIS OF THE PILOT PLANT.

## 6.1. Introduction and objective.

The main objective of this economic analysis was to determine whether this pilot plant should be installed in parallel or in series with the existing wastewater treatment plant at the Agropur's cheese factory in Notre Dame du Bon Conseil, Québec, Canada. Two options were considered in this study ;

Option A.

This involve an economic analysis of the pilot plant installed in parallel to the existing treatment plant. The same raw material ( effluent from the cheese plant ) was used in both the pilot plant and the existing treatment plant.

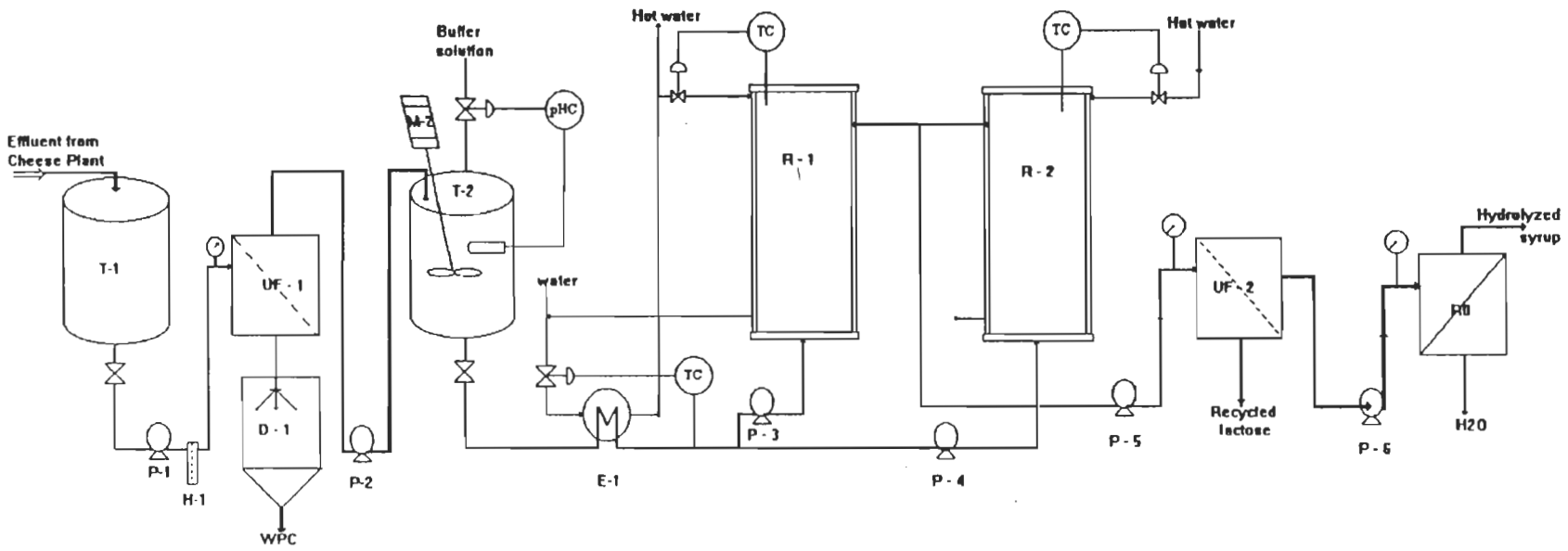
Option B.

In case B, an economic analysis of the pilot plant installed in series with the existing treatment plant was studied. In this option, the raw material for the pilot plant was the effluent from the existing treatment plant.

The process flow diagrams for option A and option B are presented in figure 32 and figure 33 respectively.

Figure32

PILOT PLANT FOR THE BIOVALORIZATION OF CHEESE INDUSTRIAL EFFLUENT.

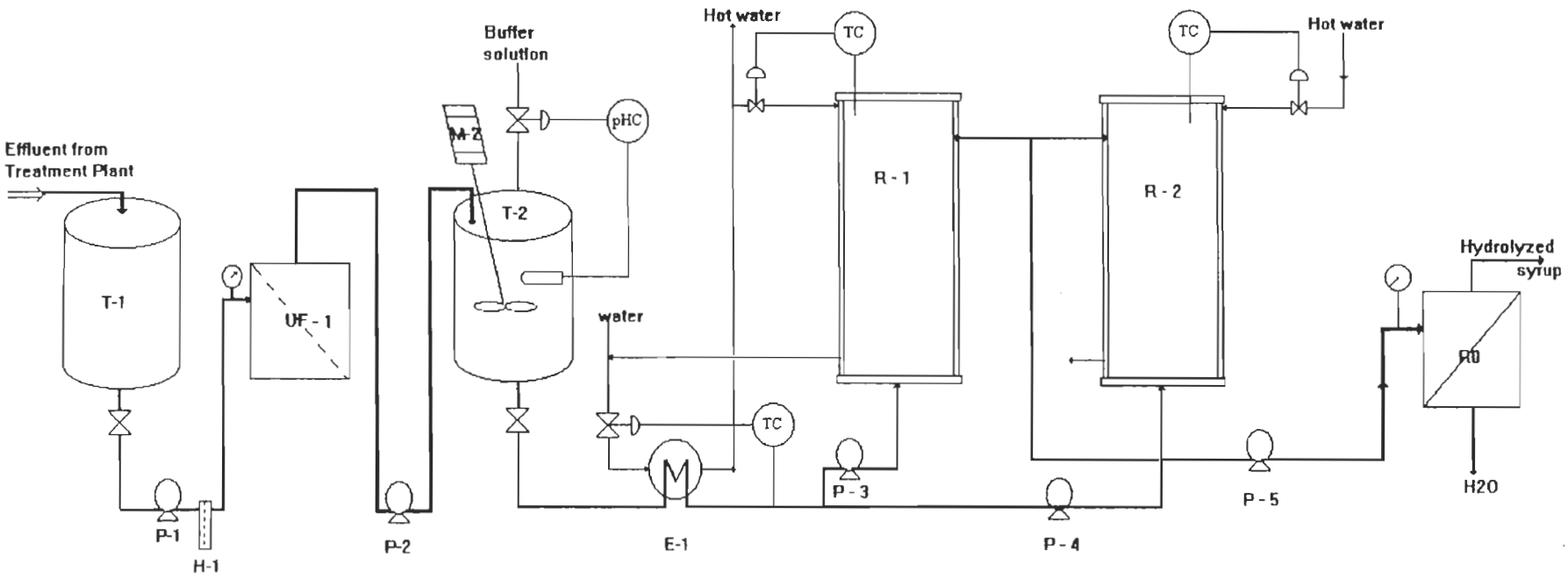



NOMENCLATURE

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| D - 1 | Spray dryer | M - 2 | Agitator | P - 3 | PFR Feed Pump | P - 6 | R-O Feed Pump |
| E - 1 | Heat Exchanger | P - 1 | Feed Pump | P - 4 | PFR Feed Pump | R - 1 | Plug Flow Reactor |
| H - 1 | Screen Filter | P - 2 | UF-1 Permeate pump | P - 5 | UF - 2 Feed Pump | R - 2 | Plug Flow Reactor |

| | | | |
|---|---|---|---|
| R - O | Reverse Osmosis | | |
| T - 1 | Pasteurization Tank | | |
| T - 2 | pH Stabilization Tank | | |
| UF | Ultrafiltration | | |

Figure33 PILOT PLANT FOR THE BIOVALORIZATION OF CHEESE INDUSTRIAL EFFLUENT.




NOMENCLATURE

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| D - 1 | Spray dryer | M - 2 | Agitator | P - 3 | PFR Feed Pump | R - 1 | Plug Flow Reactor | T - 1 | Pasteurization Tank |
| E - 1 | Heat Exchanger | P - 1 | Feed Pump | P - 4 | PFR Feed Pump | R - 2 | Plug Flow Reactor | T - 2 | pH Stabilization Tank |
| H - 1 | Screen Filter | P - 2 | UF-1 Permeate pump | P - 5 | R-O Feed Pump | R - O | Reverse Osmosis | UF | Ultrafiltration |

## 6.2. Equipment specifications.

Table 19 illustrate the principal charateristics of the equipments employed in this pilot project. The identification numbers are identical to those on the process flow diagrams.

## 6.3. Estimation of equipment cost or fixed capital.

The process flow diagrams in figure 32 and figure 33 were used to estimate the capital cost of equipment or fixed capital, $C_{FC}$. The capacity or size specification actually served as basis to determine the cost price of each major flow sheet equipment items from charts, suppliers, references, or from chemical engineering literature [72, 73, 74, 75] . The cost price of equipments obtained from charts were updated using the appropriate chemical engineering plant cost indices[76] uptil **April, 1995.**

The cost price of each equipment, and the total grass root capital or fixed capital for each option is summarized in table 20.

## Table 19:

## Equipment specifications

| Equipment identification | Identification number | Capacity or size specification |
|---|---|---|
| Heat Exchanger | E - 1 | A = 10 $m^2$ (Double pipe type) |
| UF - 1 Pump | P - 1 | ws = 1.6 kw or P = 2 hp |
| PH Tank Feed Pump | P - 2 | ws = 1.6 kw or P = 2 hp |
| Reactor Feed Pump | P - 3 | ws = 0.8 kw or P= 1 hp |
| Reactor (R - 2) Feed Pump | P - 4 | ws = 0.8 kw, P = 1 hp |
| UF - 2 feed Pump | P - 5 | ws = 1.6 kw, P = 2 hp |
| Reverse Osmosis unit Feed Pump | P - 6 | ws = 1.6 kw, P = 2 hp. |
| Plug Flow Reactor | PFR - 1 | V = 1 $m^3$, L = 3 m, D =0.65 m |
| Plug Flow Reactor | PFR - 2 | V = 1 $m^3$, L = 3 m, D =0.65 m |
| Pasteurization Tank | T - 1 | V = 60 $m^3$, L = 9 m, D =2.9 m |
| Mixing Tank with Agitator | T - 2 | V = 60 $m^3$, L = 9 m, D =2.9 m |
| Ultrafiltration unit | UF - 1 | A=20 $m^2$ Each , 5 in parallel |
| Ultafiltration Unit | UF - 2 | A = 20 $m^2$ Each, 5 in parallel |
| Spray Dryer | D - 1 | V = 30 $m^3$ |
| Reverse Osmosis Unit | R - 0 | A=50 $m^2$ Each, 2 in parallel |

**Table 20 :**

**Estimation of equipment cost, $C_E$.**

| | | Option A | | Option B | |
|---|---|---|---|---|---|
| Equipment identification number | Equipment cost $C_P$,1990x381/358 = $C_P$, 1995 | Equipment cost from suppliers & manufacturers(*, **) | TOTAL ( C$ ) | Equipment cost from suppliers & manufacturers | TOTAL ( C$ ) |
| Heat[72] Exchanger E - 1 | 16,622x381/358 = 17,690 | | 17,690 | | 17,690 |
| Pumps[*] | | | | | |
| P - 1 | | 316 | | 316 | |
| P - 2 | | 316 | | 316 | |
| P - 3 | | 269 | | 269 | |
| P - 4 | | 269 | | 269 | |
| P - 5 | | 316 | | - | |
| P - 6 | | 316 | | 316 | |
| | | 1,802 | 1,802 | 1,428 | 1,486 |
| Reactors [73] | | | | | |
| R -1 | 8,160x381/315 = 9,870 | | | | |
| R - 2 | 8,160 x 381/315 = 9,870 | | 19,740 | | 19,740 |
| Separators** | | | | | |
| UF - 1 | | 5x32640=163,200 | | 163,200 | |
| UF - 2 | | 5x32640=163,200 | | 0 | |
| R - O | | 2x68000=136,000 | 462,400 | 136,000 | 299,200 |
| Tanks [72] | | | | | |
| T - 1 | 53,040 x 381/358 = 56,448 | | | | |
| T - 2 | 100,640 x 381/358 = 107,106 | | 163,554 | | 163,554 |
| S. Dryer*** D - 1 | | 59, 185 | 59,185 | | - |
| Total Cost of Equipment | | | 724,371 | | 501,670 |

* price obtained from Piscine Launier, Trois - Rivières.

** price obtained from Koch Membrane, MA, U.S.A.

*** price obtained from Barr & Murphy Canada Ltd., Ontario.

## 6.4. Capital cost estimation.

Fixed capital or Capaital cost is the sum of direct project expense, indirect project expenses contingency and fee, start up expenses and cost of auxiliary facilities. The module factor method proposed by Gutherie [74] , is advocated, illustrated and employed in this project. This technique is accurate, direct, and relatively easy to employed. The procedure is as follows :

- Determine cost price of each major flow sheet equipment item shown in table 20.
- Update the price using appropriate cost indices up to the year that the estimate is required.
- multiply each updated cost price by the installation factor to determine the base bare module contribution of each equipment
- Sum bare module prices to obtain total bare module capital
- Since this pilot plant is to be attached to an already existing plant, multiply the total bare module capital by 1.18 to account for contingency and fee yielding the total module capital which is also the fixed capital.

This is the fixed capital required for purchasing equipments, building that houses the plant, and installation of equipments.

### 6.4.1. Installation factor, $F_{BM}$.

Installation involves direct materials and labor, indirect expenses, contingency and fee allowance, and capital for auxiliary facilities.

### 6.4.2. Direct labor and materials, $C_{DLM}$.

This includes the price of purchasing equipment, $C_E$, materials used for installation (piping, steel instruments, electrical materials, insulation and paints ), and direct labor used the installations. According to Gutherie this can be calculated as follows :

| | | |
|---|---|---|
| Cost of purchasing equipments | = | $C_E$. |
| Cost of materials | = | 0.71 $C_E$ |
| Cost of labor used for installation | = | 0.63 $C_E$. |
| Total cost direct labor and materials | = | 2.34 $C_E$ |

## 6.4.3. Indirect cost.

Indirect costs involves freight, insurance and sales taxes on purchased equipment, construction overhead and contractor engineering expenses.

#### 6.4.3.1. Construction overhead, $C_o$,

This refer to labor, fringe benefits for workers (health insurance, holidays and vacation pay, retirement benefits etc.), mandatory fees such as social security,

unemployment insurance, salaries, fees for supervisory and advisory personnel. It also includes miscellaneous items such as job side clean up and security services, warehousing, temporary buildings, roads, parking areas and other facilities required only during the construction period. Construction overhead is a function of direct labor used during installation, amounting to approximately 70 percent of the direct labor cost, $C_L$ . Therefore Overhead cost, $C_O$ is equal to 0.7 $C_L$.

#### 6.4.3.2. Engineering cost, $C_{EC}$.

This comprises salaries for project and process engineers, designers, and associated overhead. It depends on the complexity of the design and is proportional to the materials. Since this pilot plant is a simple plant, the engineering cost is estimated to be about 15 percent of direct installation cost. Thus, Engineering Cost, $C_{EC}$. is taken as 0.15 $C_{DLM}$.

#### 6.4.3.3. Contingency and fee

In order to compensate for unpredictable events, such as storms, floods, strikes, price changes, small design changes, error in estimation, and other unforeseen expenses, a contingency factor is added. For this project , a contingency factor of 10 percent of direct and indirect plant cost was chosen. Therefore, Contingency cost, $C_C$ is taken as 0.1 ($C_{DLM}$ + $C_{ID}$ ). Contractor fee varies for different situations,

but an average estimate of 3 percent of direct project and indirect project cost is acceptable. Thus, Contractor's Fee, $C_{CF}$, is taken as 0.03 ($C_{DLM}$ + $C_{ID}$) which is also equal to 0.03 $C_{BM}$.

#### 6.4.3.4. Start up expense

These are expenses incurred for changes made after the completion of a plant construction, in order to enable the plant operate at maximum design operations. These changes involved expenses for materials and equipment as well as losses in income endure while the plant is shot down or is operating at partial capacity. An allowance of 8 percent of the fixed capital is acceptable. Thus , Start-up Expenses, $C_{SE}$, is equal to 0.08 $C_{FC}$.

### 6.4.4. Auxiliary facilities.

Auxiliary facilities incorporates site development, auxiliary buildings and off site facilities.

#### 6.4.4.1. Site development

This include location of land and survey, civil engineering evaluation and site design, drainage, construction of roads, parking lots, walkways, water lines, site fencing, and sewer. Site development cost is estimated at an average value of 5 percent of the total module cost.

The Cost of Site Development, $C_{SD}$, is taken as 0.05 $C_{TM}$ or 0.19 $C_E$.

#### 6.4.4.2. Auxiliary building

This comprises administrative offices, laboratory, garage, maintenance shop, recreational rooms, warehouses as well as service buildings. The cost of auxiliary building represent approximately 4 percent of the total module cost .

#### 6.4.4.3. Off site facilities

Off site facilities are necessary to supply utilities and for convenience, safety, and pollution control. Examples include electrical power generators, raw material and final storage tanks, warehouses, fire protection utilities, yard lighting, communication networks, etc. Since this plant is to attached to an already existing plant an average value of 21 percent of the total bare module is acceptable as off site facilities cost.

Thus, Cost of Off site Facilities, $C_{OF}$, is equal to 0.21 $C_{TM}$.

Table 21 summarizes the cost capital estimation of this pilot plant.

### 6.4.5. Estimation of working capital and total capital.

Working capital consist of the total amount of money invested before there is a product to sell. This include money invested in raw materials and supplies carried

in stock, finished products in stock, semi - finished product in the process of being manufactured, cash kept on hand for monthly payment of operating expenses, accounts receivable and payable, and taxes. Since the raw material in this study is extremely cheap, a working capital of about 10 percent of the fixed capital was assumed.

Thus, working capital, $C_{WC}$ = 0.1 x $C_{FC}$

Case A : $C_{WC}$ = 0.1 x 3,359,580 = 335,958 $

Case B : $C_{WC}$ = 0.1 x 2,339,309 = 233,931 $

Total Capital is the sum of the working capital and the fixed capital, and represents the amount of money that must be provided by investors.

Total Capital, $C_{TC}$ is therefore equal to $C_{FC}$ + $C_{WC}$.

Table 22 summarizes the working capital and total capital required for this pilot plant.

Table 22

| ITEMS | OPTION A | OPTION B |
|---|---|---|
| Working capital, $C_{WC}$ | 335,958 | 233,931 |
| Total capital, $C_{TC}$ | 3,695,538 | 2,573,240 |

## Table 21. Fixed Capital Estimation.

| ITEMS | OPTION A COST (C$ ) | OPTION B COST (C$) |
|---|---|---|
| **Direct Project Expenses** | | |
| Direct Materials | | |
| Cost of Equipments, $C_E$. | 724,371 | 501,670 |
| Installation Material , $C_M = 0.71\ C_E$. | 514,303 | 356,186 |
| Direct Labor, CL = 0.63 CE | 456,354 | 316,052 |
| Total Direct labor &material Cost, CDLM | 1,695,028 | 1,173,908 |
| **Indirect Project Expenses** | | |
| Freight, Insurance & Taxes, $C_{IT}=0.07(C_E+C_M)$ | | |
| Construction Overhead,$C_O = 0.7\ C_L$ | 86,707 | 68,628 |
| Contractor Engineering Expense, $0.15(C_E+C_M)$ | 319,448 | 221,236 |
| Total Indirect Project Cost, $C_{IPC}$ | 185,801 | 128,678 |
| | 591,956 | 418,542 |
| Bare Module Capital, $C_{BM} = (C_{DLM}+C_{IPC})$ | 2,286,984 | 1,592,450 |
| Contigency, fee and Start-up Expenses | | |
| Contigency, $C_C = 0.10\ C_{BM}$ | 228,698 | 159,245 |
| Fee , $C_F = 0.03\ C_{BM}$ | 68,610 | 47,774 |
| Total Contigency & Fee , $C_{CF}$ | 297,308 | 207,019 |
| Total Module Capital, $C_{TM} = (C_{BM} + C_{CF})$ | 2,584,292 | 1,799,469 |
| Auxiliary Facilities | | |
| Site Development, $C_{SD} = 0.05\ C_{TM}$ | 129,215 | 89,973 |
| Auxiliary Buildings, $C_{AB} = 0.04\ C_{TM}$ | 103,372 | 71,979 |
| Offsite Facilities, $C_{OF} = 0.21\ C_{TM}$ | 542,701 | 377,888 |
| Total Auxiliary Cost, $C_{TAC}$ | 775,288 | 539,840 |
| **Total Grass-Root or Fixed Capital, $C_{FC}$.** | **3,359,580** | **2,339,309** |

## 6.4.6. ESTIMATION OF TOTAL PRODUCT COST.

This is divided into two categories; manufacturing cost and general expenses. In this project the annual cost basis method was used for the estimation of the total product cost. Table 5 represent a summary of the total product cost.

### 6.4.6.1 Manufacturing cost estimation.

Manufacturing costs are all expenses directly connected to the manufacturing operation. These expenses are divided into three groups :

- direct production
- fixed charges
- plant - overhead cost.

### 6.4.6.2 Direct production cost.

Direct production cost, $C_{DPC}$, involves expenditures for raw materials, cost of immobilized enzymes, direct operating labor, plant maintenance and repairs, and utilities. An operating factor, fo, of 90 percent was used in this project to convert from a one-day to a one-year basis, as well as to acknowledge that no plant can operate continuously without interruption. The operating factor represents the fraction of time that this plant is in equivalent full-scale production. This interruption time, which sums to about 16.8 hrs/week, is the time required to clean and disinfect the process equipments, changing of biocatalyst, and maintenance.

It is important to clean and disinfect processing line because microbial attack can lead to deactivation of the biocatalyst.

#### 6.4.6.2.1 Raw material

The raw material employed in this project was the effluent from cheese manufacturing industries. Due to stringent environmental regulations, small cheese manufacturers either pay processors to collect and dispose their cheese effluent or sell them to farmers as animal feed at 3.6$/m$^3$. Assuming that the cost of raw material is 3.6 $ / m$^3$, then the annual cost for raw material is 48 m$^3$/day x365 days/yr x 0.9 x 3.6 $/m$^3$ which equal to 56,765 $/yr.

#### 6.4.6.2.2 Operating labor

Since this plant was to be attached to an existing treatment plant, the equipment is assumed to require only one third (1/3) of the attention of an operator. Considering the operation is continuous, 24 hrs/day, the number of persons employed on an annual basis for this task would be 4x1/3 = 1.3 persons. Thus, annual operating labor cost, $A_{OC}$ is 4/3 x 45,000$/yr[77] x 381/358 which is equal to 63,855 $/yr.

#### 6.4.6.2.3 Cost of immobilized enzymes

The cost of immobilized enzymes was calculated from three principal items :

- cost of enzyme - *Aspergelluis oryzae.*
- cost biocatalyst carrier ( granular diatomaceous earth).
- cost chemicals utilized during the immobilization process.

The total annual cost of immobilized enzymes was calculated as indicated in Appendix 1 . Therefore, Total annual cost of IME; $C_{IME}$ is 91,366 $

#### 6.4.6.2.4 Cost of acetate buffer

Acetate buffer is used to maintain the pH of the ultrafiltration permeate at 4.5.

Annual cost of acetate buffer at 0.186 $/L

= 233L/day x 0.186 $/L x 365 days/yr x 0.9 = 14,237 $/yr

#### 6.4.6.2.5 Supervisory and clerical labor

Direct supervisory and clerical labor is always required for manufacturing operation. It is closely related to the total amount of operating labor, complexity of the operation and the production quality standard. This cost averages about 15 percent of the operating labor cost in a project like this.

Thus, supervisory and clerical labor cost, $C_{S\&Cl} = 0.15\ C_{OL}$ or 9,578 $

#### 6.4.6.2.6 Utilities

Typical utilities cost include electricity, process steam, hot water, compressed air, and refrigeration used in the plant. Utility cost for ordinary chemical processes is estimated between 10 - 20 percent of the total production cost [72]. However, it can also be calculated as shown in Appendix 2.The total annual cost for utilities was calculated to be 11,485$ .

#### 6..6.2.7 Maintenance and repairs.

This expense is necessary for the maintenance and repairs in order to keep the plant in efficient operating conditions. It include the cost for labor, materials, and supervision. Since this plant is relatively a simple process, the annual maintenance and repair cost was estimated at 6 percent of the fixed capital. Annual maintenance and repair cost, $C_{M\&R}$ is taken as 0.06 $C_{FC}$.

#### 6.4.2.6.7 Operating supplies

Many miscellaneous supplies are needed to keep the process functioning properly. This include items such as instrument charts, lubricants, cleaning chemicals and disinfectants, custodial supplies, and any other items not considered as part of regular maintenance. The annual cost of operating supplies is about 15 percent of the maintenance and repair cost.

Annual operating supplies cost, $C_{OS}$ is taken as 0.15 $C_{M\&R}$

#### 6.4.6.2.8 Laboratory expense

This cost result from quality control testing and chemical or physical analysis necessary to certify product purity and viability. The annual cost for laboratory test was estimated at 15 percent of the operating labor cost.

The annual laboratory cost, $C_L$ is 0.15 $C_{OL}$ or 9,578 $

#### 6.4.6.2.9 Patent and royalties.

They are accrued in many processes that are licensed from another firm. This cost is estimated in this project to be approximately 3 percent of the total product cost.

Annual patent and royalty costs , $C_{P\&R}$ is taken as 0.03 $A_{TE}$

### 6.4.6.3 Indirect manufacturing expenses.

These expenses are also known as fixed cost because they are invariant with the amount of production. These include costs for local property tax, insurance, depreciation, plant overhead and other miscellaneous expenses.

#### 6.4.6.3.1 Plant overhead and other miscellaneous cost.

Overhead and other miscellaneous form an important component of indirect manufacturing expenses. Prominent in this category are social security, unemployment insurance, fringe benefits, packaging, warehouses, general

engineering, salaries, etc. Plant overhead cost are based on the sum of operating labor, supervision and maintenance. About 60 percent of this sum would adequately represent the plant overhead cost in this project.

Thus annual plant overhead costs , $C_{POC}$ is equal to 0.6 ($C_{OL}$ + $C_S$ + $C_{M\&R}$)

#### 6.4.6.3.2 Local taxes

Since this plant is located in a less populated area, the local property tax was estimated at 1.5 percent of the fixed capital. Therefore, annual local taxes Cost is taken as 0.015 $C_{FC.}$

#### 6.4.6.3.3 Insurance

This is necessary to insure the plant against accidents such as fire, thief, vandalism, flood, strong winds, etc. The cost of insurance is relatively low and is estimated at 0.5 percent of the fixed capital.

#### 6.4.6.3.4 Depreciation

Depreciation is the decrease in value of an asset (physical properties) with the passage of time. Holland et al.[79] present four chemical engineering definition for depreciation:

- A tax allowance.
- A cost of operation.

- A means of building fund to finance plant replacement.
- A measure of falling value.

The straight-line method is normally used to determine the rate of depreciation in engineering projects. In this method, the difference between the initial cost and salvage value is divided by the total years of useful life to give the annual depreciation. The chemical industry has a useful life of 10 years. The salvage value is generally negligible, and the annual straight-line depreciation over a 10 years period is approximately 10 percent of the fixed capital.

Thus, the annual depreciation cost, $C_{DC}$ is 0.1 x $C_{FC}$

### 6.4.6.4 General expenses.

Besides the direct and indirect manufacturing expenses, a certain portion of corporate management cost, sales expenses, and research effort must be financed from plant revenue. Administrative costs incorporate salaries and wages for administrators, secretaries, accountants, typists, cost for office supplies and equipment, and other overhead items related to administrative activities. In this project, administrative expenses was estimated to be 25 percent of overhead cost. Therefore the annual administrative costs, $C_{AC}$ is equal to 0.25x $C_O$

#### 6.4.64.1 Research and development

In chemical and environmental industries, new products and methods are constantly being developed. Research and development cost include salaries and wages for all personnel directly concern with research and development, fixed and operating expenses for all equipments involved, cost for materials and supplies, direct overhead expenses and miscellaneous costs. Research and development cost amounts to about 5 percent of the total product cost, $A_{TE}$ .Thus, the annual cost for Research & Development is equal to 0.05 $A_{TE}$.

#### 6.4.6.4.2 Distribution and marketing cost

This is usually based product value. Prominent items in this group include cost for sales offices, salesmen expenses, shipping, advertisements, technical services, and salaries for personnel connected to this type of work. Since this product would be sold in small quantities to large numbers of customers, 10 percent of the total expense was chosen for the cost of distribution and marketing.

Annual distribution and marketing costs, $C_{DMC} = 0.1\ A_{TE}$

#### 6.4.6.5 Revenue from sales

**Option A :** The two marketable products obtained from option A are whey protein concentrate(WPC 35%), which is the retentate from the UF - 1 unit and the syrup of glucose and galactose obtained from the reverse osmosis unit. An

elaborate calculation of the annual sales revenue is shown in Appendix 3. The annual sales revenue for option A , $A_{SR1}$ is 1,610,037 $ .

**Option B :** Here the permeate contained approximately 0.2 % (w/v) of lactose and was hydrolyzed at about 99% in the bioreactors. No WPC was found in the retentate since they were almost completely fermented the anaerobic reactors of the treatment plant to produced methane. Annual revenue from the hydrolyzed syrup is (0.2 + 0.7)/100 x 47 $m^3$/d x 365 d/yr x 0.9 x 1000 L/$m^3$ x 1.4 $/L = 58,361.3 $/yr.

### 6.4.7. Profitability

The minimum attractive rate of return (MARR) is an interest rate chosen to minimized the economic well-being of a company. The criteria for estimating this interest rate is based on four factors :

- Money available for investment, source and cost of fund ( equity, borrow funds )
- Number of good projects available for investment and their purposed.

**Table 23 Summary of the Total Product Cost.**

| COMPONENTS | Option A ($ /yr.) | Option B ($ / yr.) |
|---|---|---|
| **Manufacturing Cost** | | |
| **Direct manufacturing cost** | | |
| Raw material(cheese factory effluent) | 56,765 | - |
| Immobilized enzymes cost, $C_{IME}$ | 91,366 | 91,366 |
| Cost of buffer solution | 14,237 | 14,237 |
| Operating labor cost | 63,855 | 63,855 |
| Supervisory & clerical labor ($0.15C_{OL}$) | 9,578 | 9,578 |
| **Power and Utilities** | | |
| Electricity at 0.07 $ / kw-h | 4,527 | 4,527 |
| Steam at 0.014 $ / kg | 6,958 | 6,958 |
| Maintenance & repairs ($0.06\ C_{FC}$) | 201,575 | 140,359 |
| Operating supplies ($0.15\ C_{M\&R}$) | 30,236 | 21,054 |
| Laboratory charges ($0.15\ C_{OL}$) | 9,578 | 9,578 |
| Patent and royalties ($0.03\ A_{TE}$) | 40,174 | 29,363 |
| Total direct manufact. expense, $A_{DME}$ | 528,849 | 390,875 |
| **Indirect or fixed cost** | | |
| Overhead(payroll & plant), | 165,005 | 128,275 |
| Local taxes (1.5% of fixed capital) | 50,394 | 35,090 |
| Insurance (0.5 % $C_{FC}$) | 16,798 | 11,697 |
| Total indirect manufact. cost, $A_{IMC}$ | 232,197 | 175,062 |
| Depreciation 10 % CFC), $A_{BD}$ | 335,958 | 233,931 |
| Total Manufacturing Expense | 761,046 | 565,937 |
| General Expense | | |
| Administrative cost,25% 0f overhead | 41,251 | 32,069 |
| Distribution & selling expense($0.1A_{TE}$) | 133,912 | 97,875 |
| Research & Development (5% of $A_{TE}$) | 66,956 | 48,938 |
| Total General Expense, $A_{GE}$ | 242,119 | 178,882 |
| Total Annual Expense, $A_{TE}$ | 1,339,123 | 978,749 |
| Revenue from sales, $A_{SR}$ | 1,610,037 | 58,361 |
| **Net annual profit or loss, $A_{NP}$** | **270,914** | **- 920,388** |
| Income taxes (50% $A_{NP}$), $A_{IT}$ | 135,457 | 0 |
| Net annual profit after taxes, $A_{NNP}$ | 135,457 | 0 |
| After rate of return, $i = (A_{NNP}+A_{BD})/C_{TC}$ | **12.76 %** | |

- Amount of perceived risk associated with investment opportunities available to the firm.
- Type of organization involved; government, public utility or competitive industry.

The MARR for this project was estimated at 10 %.

The most widely used rate of return method for performing engineering economic analysis is the internal rate of return (IRR) method. It solves the interest rate that equates the equivalent worth of an alternative cash inflows ( receipts or savings) to the equivalent worth of cash outflows (expenditure, including investments). It is calculated by using the present worth formulation.

$$\Sigma R_k(P/F, i\%, k) = \Sigma E_k(P/F, i\%, k)$$

where $R_K$ = net revenue; $E_k$ = net expenditure; N = project life

Once i% is calculated, it is compared with MARR to assess whether the alternative is acceptable. If i% > MARR, the alternative is acceptable ; otherwise it is not[83] .

Using the above equation i was found to be 10.2 % [ Appendix 4 ]

Since IRR is 10.2 percent and is greater than the MARR, which is 10 percent. This project is acceptable and profitable from the engineering economic analysis.

### 6.4.7.2 Calculation of rate of return.

The rate of return is also calculated by using the formula :

$$i = ((A_{NNP} + A_{BD}) / C_{TC}) \times 100$$

where $A_{NNP}$ = net profit after taxes, $A_{BD}$ = annual depreciation

$C_{TC}$ = total capital investment

$i = ((135,457 + 339,580) / 3,695,538) \times 100 = 12.8\ \%$

Here depreciation is considered as repayment on loan. The principal would decline with time and the net profit after taxes would represent the interest payment.

### 6.4.7.3 Unit sales price of hydrolyzed syrup

The unit sales price for the hydrolyzed glucose and galactose syrup was obtained by making a division of the annual sales income by the annual capacity yields.

Therefore, Unit Sales Price =1,098,586 $/yr / 13,601,542.5L/yr = 0.081 $/L

### 6.4.7.4 Payback period.

The payback period is the minimum length of time theoretically necessary to recover the original capital investment in form of cash from the project

Payback period, $P_T$ , = Total capital / Net annual revenue

= 3,695,538 / 606,872 $\approx$ 6 yrs.

# VII. CONCLUSION.

In this research it has been shown that cations play a significant role on the activity and stability of the lactase, Aspergillus oryzae. This contradicts the null effect on the activity of fungal lactase by cations as reported in literature [16,17] . However, nowhere in literature has any an experimental investigation been performed to demonstrate the null effect of cations on fungal enzymes.

Divalent cations significantly influenced the the rate of lactose hydrolysis reaction. A FT -IR spectral analysis of the effect of cations on the kinetics of lactose hydrolysis showed that they acted by increasing the binding of lactose to the enzyme as well as by increasing the maximum activity.

$Ca^{++}$ and $Mg^{++}$ were activators while monovalent cations ( $K^{+}$, $Na^{+}$ ) were stabilizers for *Aspergillus oryzae*. However, these cations may become toxic when their concentration are beyond certain limits. Consequently, it is very important to control cation concentrations in the reaction medium. An optimum concentration of 75mM NaCl : 75mM KCl : 0.5mM $CaCl_2$ was observed for the combined cations studied in this research, corresponding to an optimum maximum rate of reaction of 6.63 $mmol.L^{-1}.min^{-1}.g^{-1}_{IME}$ and a $K_{m.app.}$ value of 72.69 mmol./L

From the spectroscopic properties of D-lactose with alkali and alkaline earth metals studied here, the following conclusion may be reached :

- The strong hydrogen bonding system of the free D - lactose is preserved upon metalation;
- There is some interaction between the metal ions and the OH, as well as the C - O groups of the D - lactose anion.

The Plug flow tubular reactor performed better and was more economical than the continuous stirred tank reactor for hydrolyzing cheese industrial effluent.

A wide range of applications are available to the hydrolyzed lactose syrup in toffee, ice cream, soft fruit drinks, yogurts, bakery and dairy desserts due of their sweetness and special protein value[72] .

It can be concluded from the economic analysis made in this study that the pilot plant proposed to be installed in parallel to the existing treatment plant at the cheese factory in Notre-Dame du Bon Conseil, Québec, Canada was profitable. This profitability was due to the fact that two valuable by-products were obtained from this option namely whey protein concentrates ( WPC 35% ) and the hydrolyzed whey syrup of glucose and galactose.

The second option which was to install the pilot in series with the existing treatment plant wasn't profitable according to my economic evaluation. This was due to the fact that only one by-product (glucose and galactose syrup) was obtained in this option. Moreover, the permeate had a low lactose content (0.2% or 2 mg/L). This was evident since most of organic matter in the effluent had undergone anaerobic fermentation in the existing treatment plant to produced methane gas.

Both options had one common advantage that they were environmentally safe projects. The Biochemical Oxygen Demand ( $BOD_5$ ) was reduced from 35,000 mg/L for cheese industrial effluent to 300 mg/L for the hydrolyzed effluent while that of the existing treatment plant was reduced from 2,300 ppm to 84 ppm well below the limit of 1,000 mg/L stipulated by Environment Canada for dairy industries.

# VI. RECOMMENDATIONS.

1. The highest maximum rate of reaction , $V_{max.}$, in the presence of single cations was obtained with $Mg^{++}$. It would be interesting to further investigate the combine effect of $Mg^{++}$ and the other three cations tested in research on the kinetic parameters of lactose hydrolysis reaction. This wasn't done in this study because of the amount of experiments involved.

2. Further interest should include more FT-IR studies on the interactions of metal ions with sugar (lactose) and if possible the mechanism proposed in this study should be confirmed by conductivity, x - ray diffraction as well as NMR studies.

3. I strongly recommend that the UF permeate; 5% (w/v) lactose should be concentrated in a reverse osmosis system to about 15 or 20% (w/v) lactose before sending it to the reactor. This would reduced a substantial amount of waste water that would have been otherwise pumped into the reactor thereby making the system more efficient and most important more profitable.

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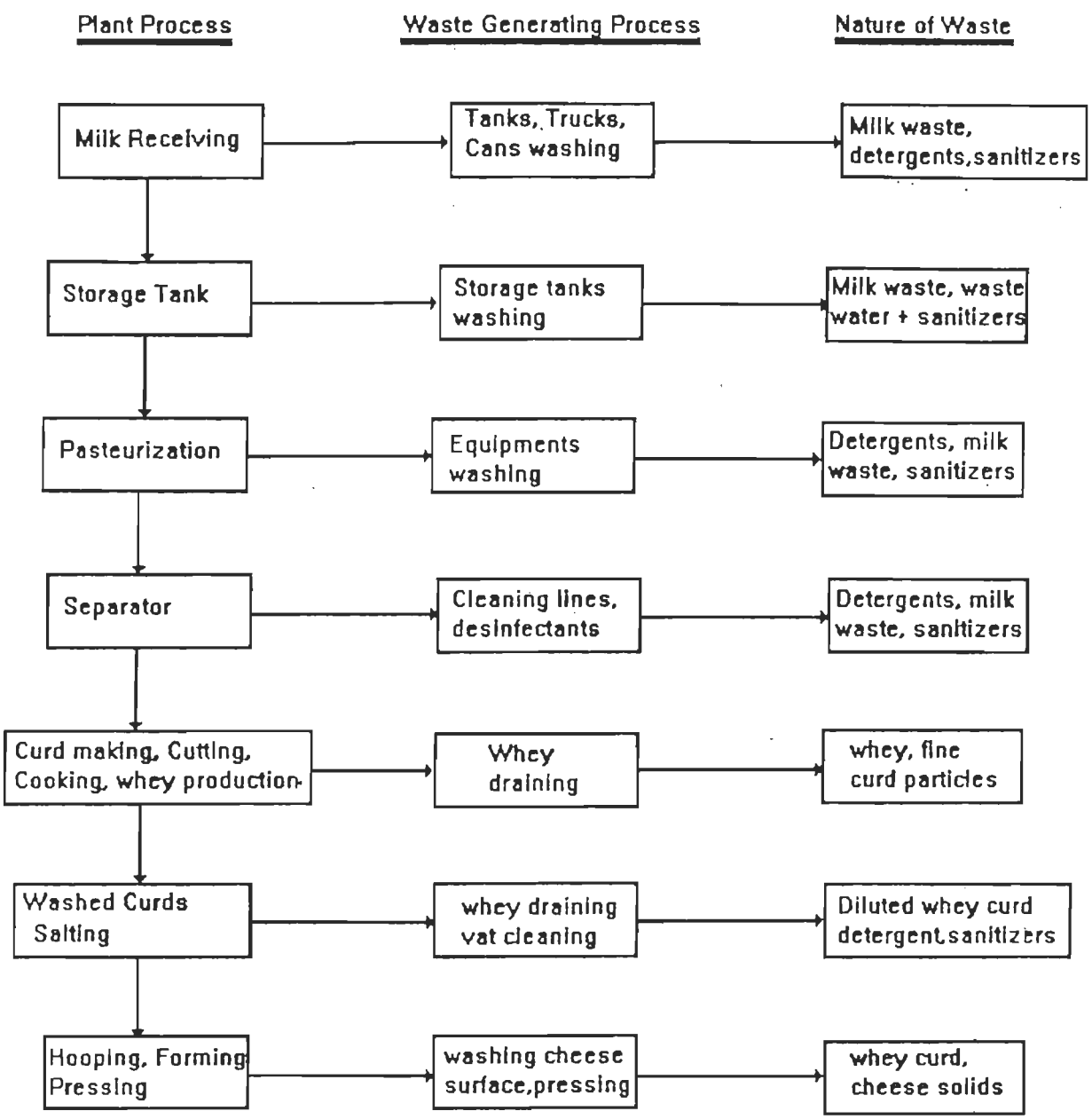
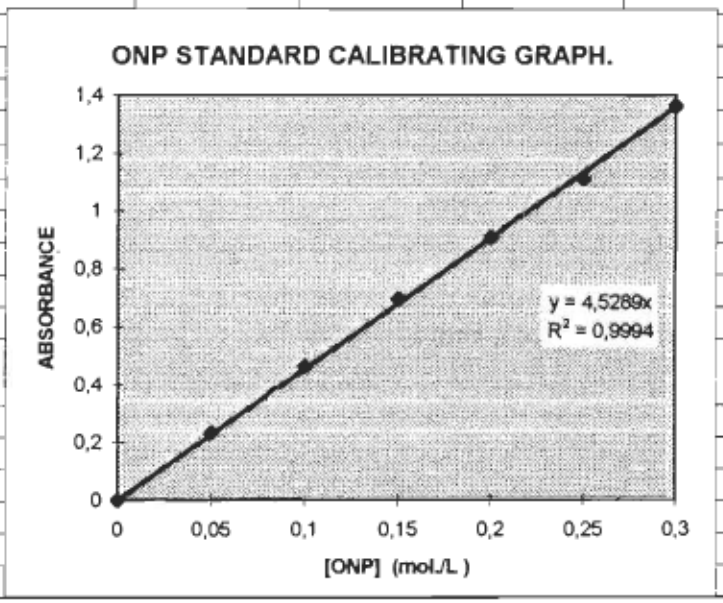
## Appendix 1 : Flow diagram for waste Generation in Agropur's Cheese Factory at Notre-Dame du Bon Conseil.



# APPENDIX 2.

STANDARD CALIBRATION CURVE OF ONP

| [ONP] | ABSORBANCE |
|---|---|
| 0 | 0 |
| 0,05 | 0,2319 |
| 0,1 | 0,4633 |
| 0,15 | 0,6954 |
| 0,2 | 0,9066 |
| 0,25 | 1,1127 |
| 0,3 | 1,362 |



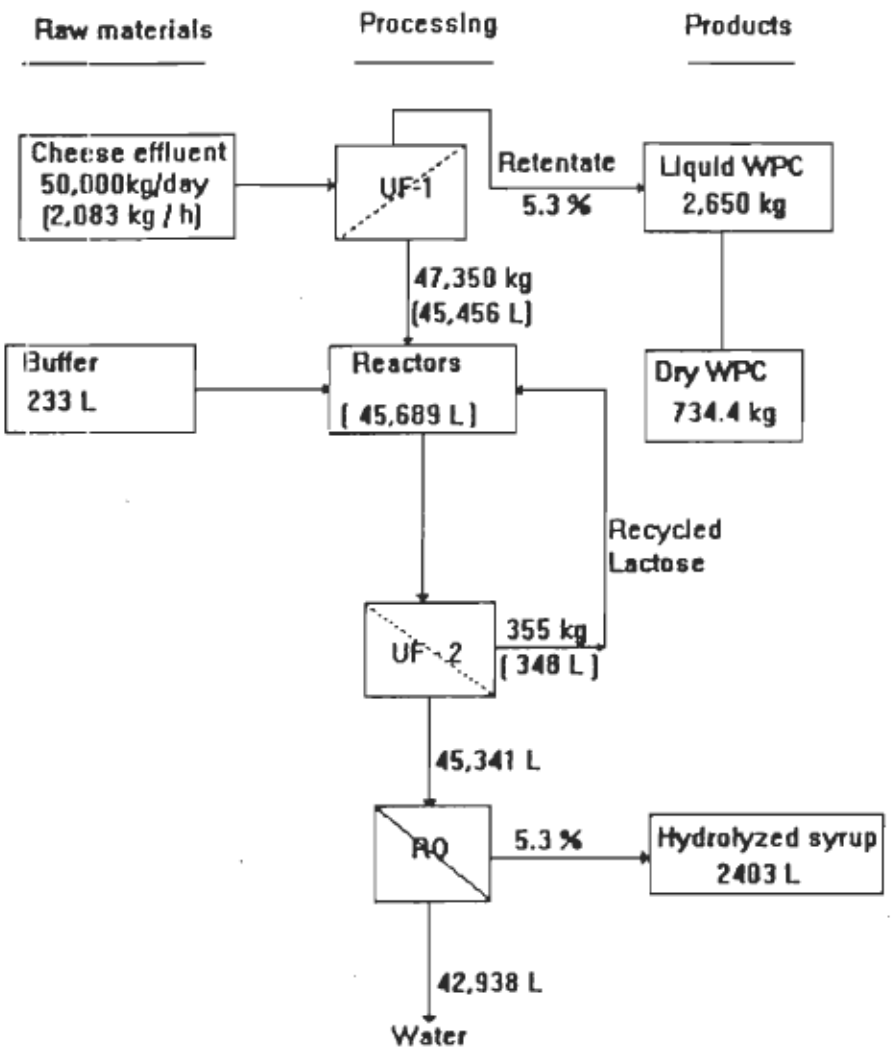
# APPENDIX 3

| TIME | LACTOSE | [S] / 360.3 | GLUCOSE | [Glu] /180.2 | GALACTOSE | [Gal] / 180.2 | CONVERSION |
|---|---|---|---|---|---|---|---|
| (min.) | [S] ( g / L ) | [S] (mol./L) | [Glu.] ( g / L ) | [Glu] (mol./L) | [Gal] ( g / L ) | [Gal] (mol./L) | ( % ) |
| T = 30 oC | | | | | | | |
| 0 | 20 | 0,055509298 | 0 | 0 | | 0 | 0 |
| 15 | 7,945 | 0,022051069 | 2,081 | 0,01154828 | 1,715 | 0,009517203 | 48,85706796 |
| 30 | 11,22 | 0,031140716 | 3,625 | 0,020116537 | 3,35 | 0,018590455 | 55,41626658 |
| 45 | 9,857 | 0,027357757 | 4,461 | 0,024755827 | 4,168 | 0,023129856 | 63,64100655 |
| 60 | 7,71 | 0,021398834 | 4,811 | 0,026698113 | 4,548 | 0,025238624 | 70,82066188 |
| 90 | 5,926 | 0,016447405 | 5,199 | 0,028851276 | 4,878 | 0,027069922 | 77,27273414 |
| 120 | 4,241 | 0,011770747 | 5,634 | 0,031265261 | 5,297 | 0,029395117 | 83,74904881 |
| 150 | 3,495 | 0,00970025 | 6,964 | 0,038645949 | 6,686 | 0,037103219 | 88,64796263 |
| 210 | 2,282 | 0,006333611 | 8,695 | 0,048251942 | 8,625 | 0,047863485 | 93,81779371 |
| | | | | | | | |
| T = 40 | | | | | | | |
| 0 | 20 | 0,055509298 | 0 | 0 | 0 | 0 | 0 |
| 15 | 11,57 | 0,032112129 | 2,864 | 0,015893452 | 2,663 | 0,014778024 | 48,85268404 |
| 30 | 8,29 | 0,023008604 | 3,996 | 0,022175361 | 3,783 | 0,020989846 | 65,23004553 |
| 60 | 4,792 | 0,013300028 | 4,921 | 0,027308546 | 4,758 | 0,026403996 | 80,15293567 |
| 90 | 3,181 | 0,008828754 | 5,118 | 0,028401776 | 4,98 | 0,02763596 | 86,38934547 |
| 120 | 2,738 | 0,007599223 | 6,884 | 0,038201998 | 6,762 | 0,037524972 | 90,88015112 |
| 150 | 2,117 | 0,005875659 | 7,394 | 0,041032186 | 7,342 | 0,040743618 | 93,29656469 |
| 180 | 1,941 | 0,005387177 | 9,496 | 0,052697003 | 9,515 | 0,052802442 | 95,14172473 |
| | | | | | | | |
| T=50 | | | | | | | |
| 0 | 20 | 0,055509298 | 0 | 0 | 0 | 0 | 0 |
| 5 | 15,97014257 | 0,04432457 | 2,1951964 | 0,012182 | 1,83472432 | 0,0101816 | 33,53458342 |
| 10 | 13,17894531 | 0,0365777 | 3,589584 | 0,01992 | 3,230986 | 0,01793 | 50,8547221 |
| 15 | 11,15964396 | 0,0309732 | 4,420306 | 0,02453 | 4,2399258 | 0,023529 | 60,80939162 |
| 30 | 7,608 | 0,021115737 | 6,231316 | 0,03458 | 6,7005568 | 0,037184 | 77,26550743 |
| 60 | 4,429 | 0,012292534 | 7,8214008 | 0,043404 | 7,74946496 | 0,0430048 | 87,54572659 |
| 90 | 3,27217254 | 0,0090818 | 8,416241 | 0,046705 | 8,30804892 | 0,0461046 | 91,08678456 |
| 120 | 2,641 | 0,007330003 | 8,7013174 | 0,048287 | 8,657516186 | 0,04804393 | 92,92886666 |
| 150 | 2,086180236 | 0,00579012 | 8,993782 | 0,04991 | 8,919968476 | 0,04950038 | 94,49610981 |
| 180 | 1,721 | 0,004776575 | 9,1948852 | 0,051026 | 9,08507132 | 0,0504166 | 95,5030953 |
| | | | | | | | |
| T=55 | | | | | | | |
| 0 | 20 | 0,055509298 | 0 | 0 | 0 | 0 | 0 |
| 10 | 13,77 | 0,038218152 | 4,778 | 0,026514983 | 4,374 | 0,02427303 | 57,06123098 |
| 30 | 7,267 | 0,020169303 | 4,431 | 0,024589345 | 4,26 | 0,0236404 | 70,51230401 |
| 45 | 4,642 | 0,012883708 | 4,6 | 0,025527192 | 4,493 | 0,024933407 | 79,66082737 |
| 60 | 4,247 | 0,011787399 | 5,829 | 0,032347392 | 5,67 | 0,031465039 | 84,40816681 |
| 90 | 3,281 | 0,0091063 | 7,318 | 0,040610433 | 7,194 | 0,039922309 | 89,84114496 |
| 120 | 2,592 | 0,007194005 | 7,931 | 0,044012209 | 7,869 | 0,043668147 | 92,4173349 |
| 150 | 2,138 | 0,005933944 | 8,821 | 0,048951165 | 8,815 | 0,048917869 | 94,28345503 |
| 180 | 1,611 | 0,004471274 | 8,352 | 0,046348502 | 8,426 | 0,046759156 | 95,41778759 |
| | | | | | | | |
| T=60 | | | | | | | |
| 0 | 20 | 0,055509298 | 0 | 0 | 0 | 0 | 0 |
| 5 | 11,9 | 0,033028032 | 2,143 | 0,011892342 | 1,919 | 0,010649279 | 40,5646236 |
| 10 | 10,78 | 0,029919512 | 2,669 | 0,014811321 | 2,54 | 0,01409545 | 49,1392102 |
| 30 | 6,058 | 0,016813766 | 3,575 | 0,019839068 | 3,448 | 0,019134295 | 69,86085046 |
| 45 | 5,093 | 0,014135443 | 4,639 | 0,025743618 | 4,52 | 0,025083241 | 78,24054519 |
| 60 | 4,249 | 0,01179295 | 4,821 | 0,026753607 | 4,714 | 0,026159822 | 81,77467145 |
| 90 | 3,727 | 0,010344158 | 6,509 | 0,036120977 | 6,385 | 0,035432852 | 87,3694604 |
| 120 | 3,126 | 0,008676103 | 7,085 | 0,039317425 | 6,981 | 0,038740289 | 89,99686217 |
| 135 | 2,819 | 0,007824036 | 6,957 | 0,038607103 | 6,918 | 0,038390677 | 90,77591601 |

# APPENDIX 4

| TIME (min.) | LACTOSE,[S] | [S]/360,3 | GLUCOSE | [GLU.]/180,2 | GALACTOSE | [GAL.]/180,2 | CONVERSION |
|---|---|---|---|---|---|---|---|
| Na | ( g/L ) | ( mol./L ) | ( g/L ) | ( mol./L ) | ( g/L ) | ( mol./L ) | ( % ) |
| 0 | 20 | 0,055509298 | 0 | 0 | 0 | 0 | 0 |
| 5 | 13,62 | 0,037801832 | 1,029 | 0,005710322 | 0,3752 | 0,002082131 | 17,09085446 |
| 10 | 12,45 | 0,034554538 | 1,434 | 0,007957825 | 0,8407 | 0,004665372 | 26,75668221 |
| 30 | 10,03 | 0,027837913 | 2,4 | 0,013318535 | 2,065 | 0,011459489 | 47,09224183 |
| 60 | 6,711 | 0,018626145 | 3,012 | 0,016714761 | 2,8 | 0,015538291 | 63,39143312 |
| 90 | 6,515 | 0,018082154 | 4,067 | 0,022569367 | 3,823 | 0,021215316 | 70,77246146 |
| 120 | 5,431 | 0,01507355 | 4,575 | 0,025388457 | 4,384 | 0,024328524 | 76,73494939 |
| 150 | 3,658 | 0,010152651 | 4,936 | 0,027391787 | 4,826 | 0,026781354 | 84,21682761 |
| K | | | | | | | |
| 0 | 20 | 0,055509298 | 0 | 0 | 0 | 0 | 0 |
| 5 | 13,34 | 0,037024702 | 1,102 | 0,006115427 | 0,479 | 0,002658158 | 19,15701562 |
| 10 | 12,59 | 0,034943103 | 1,571 | 0,008718091 | 1,029 | 0,005710322 | 29,22416423 |
| 30 | 10,01 | 0,027782404 | 2,546 | 0,014128746 | 2,267 | 0,012580466 | 49,01526909 |
| 60 | 7,61 | 0,021121288 | 3,386 | 0,018790233 | 3,172 | 0,017602664 | 63,27638484 |
| 90 | 6,397 | 0,017754649 | 4,185 | 0,023224195 | 3,96 | 0,021975583 | 71,79761651 |
| 120 | 4,651 | 0,012908687 | 4,439 | 0,02463374 | 4,26 | 0,0236404 | 78,90145352 |
| 180 | 3,508 | 0,009736331 | 4,877 | 0,027064373 | 4,785 | 0,026553829 | 84,63199017 |
| Ca | | | | | | | |
| 0 | 20 | 0,055509298 | 0 | 0 | 0 | 0 | 0 |
| 5 | 7,976 | 0,022137108 | 1,976 | 0,010965594 | 1,696 | 0,009411765 | 47,93041096 |
| 10 | 6,546 | 0,018168193 | 2,788 | 0,015471698 | 2,7 | 0,014983352 | 62,63475653 |
| 15 | 5,054 | 0,0140272 | 3,53 | 0,019589345 | 3,449 | 0,019139845 | 73,41137305 |
| 30 | 2,894 | 0,008032195 | 4,281 | 0,023756937 | 4,256 | 0,023618202 | 85,5033715 |
| 60 | 1,5999 | 0,004440466 | 5,699 | 0,031625971 | 5,808 | 0,032230855 | 93,49832746 |
| 90 | 1,009 | 0,002800444 | 5,73 | 0,031798002 | 5,9 | 0,032741398 | 95,8413268 |
| 150 | 0,7743 | 0,002149042 | 6,432 | 0,035693674 | 6,667 | 0,03699778 | 97,12850319 |
| Mg | | | | | | | |
| 0 | 20 | 0,055509298 | 0 | 0 | 0 | 0 | 0 |
| 5 | 1,53 | 0,004246461 | 1,924 | 0,010677026 | 1,608 | 0,008923418 | 82,19282024 |
| 10 | 8,232 | 0,022847627 | 2,9 | 0,01609323 | 2,798 | 0,015527192 | 58,05315683 |
| 15 | 6,218 | 0,017257841 | 3,566 | 0,019789123 | 3,471 | 0,019261931 | 69,35148405 |
| 30 | 3,466 | 0,009619761 | 4,602 | 0,025538291 | 4,575 | 0,025388457 | 84,11178215 |
| 60 | 1,893 | 0,005253955 | 5,667 | 0,031448391 | 5,761 | 0,031970033 | 92,34924563 |
| 90 | 1,226 | 0,00340272 | 6,976 | 0,038712542 | 7,13 | 0,039567148 | 95,83420716 |
| 150 | 0,7133 | 0,001979739 | 7,513 | 0,041692564 | 7,871 | 0,043679245 | 97,73359586 |

Appendix 5 : Material Balance for One Operating Day



# APPENDIX 6.

**Cost of enzyme**

A. oryzae was purchased from Sigma chemicals at 0.99 $/g. For the pilot plant the quantity of enzymes required per reactor per cycle was calculated to give 1,438g

This implies that cost of enzymes = 0.99$/g x 1,438g = 1,429 $/reactor/cycle

Total cost of enzymes for the two reactors per cycle = 2 x 1,429 = 2,858$

A cycle is define as the period of time the enzyme is used before replacement.

**Cost of biocatalyst carrier (support)**

Granular diatomaceous earth was obtained from Eagle-Picher minerals, Inc. at 26.92 $/kg

Cost of GDE per reactor per cycle = 1,514 $.

Total cost of GDE for two reactors = 2 x1514 = 3,028 $/cycle

**Cost of chemicals**

the following chemicals were used :

| | |
|---|---|
| Glutaraldehyde | 323.35 $/reactor/cycle |
| Polyethylenimine (PEI) | 202.05$ /reactor/cycle |
| Sodium hydrogen Carbonate($NaHCO_3$) | 8.2 $ /reactor/cycle |
| Phosphate buffer at 0.393$/L | 282.57 $/reactor/cycle |

| | |
|---|---|
| Cost of chemicals | 816 $/reactor /cycle |
| **Total cost of chemicals** | **2 x 816 = 1,632 $ /cycle.** |

Therefore cost of immobilization per cycle = 7,518 $

The half-life of the biocatalyst used in this studies was estimated at 17 days. Assuming that the biocatalyst was replaced when its activity decreased to 50 percent of the original activity which is 17 days; then the biocatalyst would be replaced 19 times per year.

Number of IME replacement per year = 365/17 x 0.9 = 19

Annual cost for chemicals and biocatalyst will be equal to :

(2,858 + 1,632) x 19 = 85,310 $/yr.

It is presumed that the biocatalyst carrier would be re-used. In order to economize time, two (2) sets of biocatalyst carrier would be required for each reactor. When one set is in the bioreactor, the other set is clean, dried and ready to be immobilized with enzyme for the next replacement. This give a total of four (4) sets of biocatalyst carriers annually for the two bioreactors.

The annual cost for biocatalyst carrier at 1514$/reactor will be equal to :

4 x 1,514 = 6,056 $/yr

Therefore, the total annual cost of IME = 85,310 + 6,056 = 91,366 $/yr.

## Appendix 7 : Calculation of utility cost.

### Electricity

The projected cost for electricity (industrial) obtained from Hydro- Québec is 0.07 $/kw-h.

Electricity is consumed mostly by the pumps and the heating system.

Cost of power consumption by all the 2 hp pumps (P-1, P-2, P-5, and P-6)

= 4pumps x 2hp/pump x 0.7457 kw/hp x 8760 h/yr x 0.07 $/kw-h x 0 .9

= 3,292 $/yr

Cost of power consumption by all the 1 hp pumps(P-3, and P-4) and the agitator is equal to 3 x 1 hp x 0.7457 kw/hp x 8760 h/yr x 0.07 $/kw-h x 0.9

= 1,235 $/yr

### Cost of steam

$$Q = m_h \times C_{p,h} \times T_h = m_c \times C_{p,c} \times T_c$$

$$= m_h \times 4180 j/kg\text{-}k \times 73^{\circ}k = 0.0171 kg/s \times 1{,}251 j/g\text{-}k \times 25\ ^{\circ}k$$

$$= m_h = 53{,}480.5 / 305{,}148\ kg/s = 0.1753\ kg/s$$

Annual steam cost, $A_S$ = 0.1753kg/s x (3.15 x 106 s/yr) x 0.9 x 0.014 $/kg [78]

= 6,958 $/yr

The total annual cost for utilities = 3,292 + 1,235 + 6,958 = 11,485 $/yr.

## Appendix 8. Calculation of annual sales revenues for option A

### Whey protein concentrate.

WPC 35% is produced from the UF system at the rate of 15.3 kg / $m^3$ of effluent[8] . WPC 35% cost 2.00$ / kg in 1993.Therefore, the cost price of WPC 35% in 1995 can be calculated to give : 2.00 x 381/360 = 2.12 C$

Annual WPC 35% revenue, $A_{WPC}$ = 15.3kg/$m^3$ x 48 $m^3$/day x 365days/yr x 0.9 x 2.12 $/kg = 511, 451 $/yr

### Glucose & galactose syrup

In this research, 1 $m^3$ of UF-1 permeate was found to contain 5% (w/v) of lactose and this lactose was hydrolyzed at 85% in the reactors. The remaining 15% was recycled. Proteins and minerals were estimated at 0.3% and 0.7 %[80, 81] respectively in the permeate. Presently, the cost of glucose & galactose syrup on the market [82] is 1.4$/L.

Annual cost of syrup, $A_{GS}$ = ((5/100 x 0.85) + (0.3%+0.7%))x 45.5$m^3$/d x 365d/yr x 0.9x 1000L/$m^3$ x 1.4$/L = 1,098,586 $

Total annual sales revenue, $A_{SR}$ = 511,451 + 1,098,586 = 1,610,037 $

## Appendix 9 Calculation of internal rate of return (IRR).

It is calculated by using the present worth formulation.

$$\Sigma R_k(P/F, i\%, k) = \Sigma E_k(P/F, i\%, k)$$

where $R_K$ = net revenue

$E_k$ = net expenditure

N = project life

$\Rightarrow \quad \Sigma(R_k-E_k)(P/F, i, k) = 0$

$\Rightarrow \quad (R - E)_k(P/A, i, 10) + S(P/F, i, 10) - I = 0$

$\Rightarrow \quad (1,610,037 - 1,003,165)(P/A, i, 10) + 0 - 3,695,538 = 0$

$\Rightarrow \quad (P/A, i, 10) = 3,695,538 / 606,872 = 6.0895$

let i = 10 % , then (P/A, i %, 10) = 6.1446

Let i = 12%, then (P/A, i%, 10) = 5.6502

Therefore, i = 10.2 %

The values of P/A are obtained from Table C - 9 in Engineering Economy by E. Paul Degarmo et al., [83] .