DETECTION OF BIFIDOBACTERIA BREVE BY INDIRECT ENZYMELINKED IMMUNOSORBENT ASSAY (ELISA)

TEE SIEW CHOON

FSMB 2001 15
DETECTION OF *BIFIDOBACTERIA BREVE* BY INDIRECT ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

TEE SIEW CHOON

MASTER OF SCIENCE
UNIVERSITI PUTRA MALAYSIA

2001
DETECTION OF BIFIDOBACTERIA BREVE BY INDIRECT ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

By

TEE SIEW CHOON

Thesis Submitted in Fulfilment of the Requirement for the Degree of Master of Science in the Faculty of Food Science and Biotechnology
Universiti Putra Malaysia

August 2001
DEDICATION

Especially dedicated to my Beloved Mum, Khor, Sister & Brothers
In this study, the suitability of fructose-6-phosphate phosphoketolase (F6PPK) from *Bifidobacterium breve*, and intact *B. breve* cells were examined as immunogens for the production of polyclonal antibodies that could be used to detect the presence of the organism. F6PPK is the enzyme that catalyzes the breakdown of fructose-6-phosphate to yield erythrose-4-phosphate and acetyl phosphate through the bifid shunt. It represents an interesting candidate as it is the key enzyme found restricted to bifidobacteria, and there are differences in molecular masses of the enzyme among human and animal sources. The study involved two stages. In the first stage, F6PPK extracted from *B. breve* was partially purified by sequential acetone-fractionation, ion-exchange chromatography and gel filtration chromatography, and then characterized. F6PPK was eluted from the anion exchanger column at 0.48 mM NaCl. Following purification by the gel filtration chromatography, the specific activity of F6PPK was found to be 11.05 fold but only 0.4% was recovered. The enzyme was not purified to homogeneity since 2 protein bands were observed on native polyacrylamide gel electrophoresis. The apparent molecular mass of the enzyme as determined by gel filtration chromatography on Superose 12 was 200,000 Daltons. The enzyme was stable, at least for 10 minutes, between 20-50°C. The optimum temperature was 37°C. It has a pH optimum of pH 6.0.
F6PPK is not a metalloenzyme since EDTA did not reduce its activity. Cations like magnesium and calcium affected the activity of the enzyme. The thiol inhibitors L-cysteine and hydroxylamine HCl, were strongly inhibited activity of F6PPK by 19.8% and 17.1% respectively. The affinity constant, $K_m$, of F6PPK was $2 \times 10^{-1}$ mM and maximal velocity, $V_{max}$, was 20 μmole/min with fructose-6-phosphate as the substrate. In the second stage of this study, the production of polyclonal antibodies was achieved by injecting F6PPK obtained after gel filtration and intact cells of B. breve intradermally into 5 New Zealand White Rabbits, three of the rabbits were injected with F6PPK. Booster doses made in Freund Incomplete Adjuvant were administered every two weeks for a total of three times. Blood was collected once before the first injection and twice after third booster and labeled as Bleed 1, Bleed 2 and Bleed 3, respectively. Assessments of the antisera were carried out by Noncompetitive Indirect ELISA. Bleed 2 contained the highest level of antibody. Extensive checkerboard titration was performed; the optimum antiserum dilution for anti-F6PPK antiserum was 1:1600, while the anti-B. breve antiserum dose-response was 1:800 and conjugate antibody was used at a dilution of 1/4000. The best absorption for both antisera were obtained using phosphate buffered saline pH 7.2 as the coating buffer. Crude extract was good enough to bind to the anti-F6PPK antiserum. Both antisera were capable of detecting bifidobacteria at the species level at $1 \times 10^5$ CFU/ml or greater. Both antisera were found to cross-react with the different strains of B. breve. A slight cross-reaction occurred with other Bifidobacterium spp. whereas they showed no significant cross-reactivity towards Lactococcus spp., Lactobacillus spp., probiotics, yogurt starter cultures and E. coli. ELISA described herein should allow unequivocal identification of B. breve.
PENGUNAAN IKATAN-ENZIM IMMUNOSORBAN ASSEI (ELISA) SECARA TIDAK LANGSUNG UNTUK MENGESAN KEHADIRAN *BIFIDOBACTERIUM BREVE*.

Oleh

TEE SIEW CHOON

Ogos 2001

Pengerusi: Profesor Hasanah Mohd Ghazali, Ph.D.

Fakulti: Sains Makanan dan Bioteknologi, UPM

Dalam kajian ini, kesesuaian enzim fruktosa-6-fosfat fosfoketolase (F6PPK) dari *Bifidobacterium breve* dan sel utuh *B. breve* sebagai imunogen untuk menghasilkan antibodi poliklonal yang boleh digunakan untuk mengesan kehadiran organism tersebut telah dijalankan. F6PPK adalah enzim yang memangkin penguraian fruktosa-6-fosfat kepada eritrosa-4-fosfat dan asetil fosfat melalui "bifid shunt". F6PPK merupakan calon yang menarik kerana ia adalah enzim yang terhad kepada spesis bifidobakteria sahaja, dan juga berat molekulnya adalah berlainan bergantung kepada samada daripada sumber manusia atau haiwan. Kajian ini melibatkan dua peringkat. Pada peringkat awal, F6PPK diekstrak dari sel *B. breve* dan ditulinkan melalui pemendapan acetone, kromatografi penukaran anion dan kromatografi penurasan gel. Selepas itu, ciri-ciri enzim ditentukan. F6PPK dielusikan dari turus penukaran anion pada gradien garam yang rendah (0.48 mM NaCl). Teknik ini membaikkan darjah penulinan sebanyak 11.05 kali tetapi hanya 0.4% enzim diperolehi selepas kromatografi penurasan gel. Enzim F6PPK tidak berjaya ditulinkan secara homogenus kerana elektroforesis gel poliakrilamid semulajadi menunjukkan dua jalur protein. Berat molekul F6PPK yang ditentukan melalui kromatografi penurasan gel Superose 12 adalah 200,000 Daltons. Enzim ini didapati stabil pada 20-50°C selama 10 minit. Suhu optimumnya adalah 37°C. Enzim ini juga
ACKNOWLEDGEMENTS

First, I would like to express my sincere thanks and deepest appreciation to my supervisor, Prof Dr. Hasanah Mohd Ghazali, for the invaluable guidance, constructive criticism, encouragement and tremendous patience throughout my research work. I have been trained to be more independent and mature either in the many aspects of my life or in my research work.

I would like to also extend my thanks to my co-supervisor Assoc. Professor Dr. Yazid Abd. Manap for his kindness for providing the ‘fascinating’ organism, bifidobacteria, sharing of knowledge and also his generosity in allowing me to use his laboratory.

Not to be forgotten, I also wish to express my heartiest gratitude to my co-supervisor Assoc. Professor Dr. Abdul Manaf Ali for his unflagging support, valuable suggestion and discussion, technical guidance and generosity to allow me to use the equipment in his laboratory. His research spirit being the most rewarding one.

My genuine thank is also forwarded to the Faculty of Food Science and Biotechnology, UPM and the Malaysian Government in providing me with a fellowship through PASCA scheme.

Thanks to all my teachers throughout my life, who have taught the alphabet as simple as ABC......, number and also the simple concept of Science. I would like to take this opportunity to thank the lecturers in the Department of Biotechnology who had
build up my fundamentals knowledge of Biotechnology during my undergraduate studies.

Special thanks are conveyed to Dr. Lai Oi Ming who had been very kind and helpful. I am also very grateful to all my friends at Enzyme Laboratory especially Chu, Pauline, ....for their kindness, assistance, guidance and help from time to time. My sincere thanks to Mr Shaharuddin from Animal House, UPM who had sacrificed of time to help me in injecting and bleeding.

Last but not the least, I am greatly indebted to my beloved Khor, mother, sister and brothers, for their unconditional loves, concerns, encouragement, support ....and effort to make me being a better person from moment to moment.
I certify that an Examination Committee met on 16th August 2001 to conduct the final examination of Tee Siew Choon on his Master of Science thesis entitled "Detection of Bifidobacterium breve by Indirect Enzyme-Linked Immunosorbent Assay (ELISA)" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

ZAITON HASSAN, Ph.D.
Department of Food Science,
Faculty of Food Science and Biotechnology,
Universiti Putra Malaysia
(Chairman)

HASANAH MOHD.GHAZALI, Ph.D.
Professor
Department of Biotechnology,
Faculty of Food Science and Biotechnology,
Universiti Putra Malaysia
(Member)

ABDUL MANAF ALI, Ph.D.
Associate Professor
Department of Biotechnology,
Faculty of Food Science and Biotechnology,
Universiti Putra Malaysia
(Member)

MOHD.YAZID ABDUL MANAP, Ph.D.
Associate Professor
Department of Food Technology
Faculty of Food Science and Biotechnology,
Universiti Putra Malaysia
(Member)

MOHD.GHAZALI MOHAYIDIN, Ph.D.
Professor
Deputy Dean of Graduate School
Universiti Putra Malaysia
Date: 11 SEP 2001
This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Science.

AINI IDRIS, Ph.D.
Professor
Dean of Graduate School
Universiti Putra Malaysia.
Date:

08 NOV 2001
DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

Name: Tee Siew Choon
Date:
TABLE OF CONTENTS

DEDICATION ii
ABSTRACT iii
ABSTRAK v
ACKNOWLEDGEMENTS vii
APPROVAL SHEETS ix
DECLARATION FORM xi
LIST OF TABLES xv
LIST OF FIGURES xvi
LIST OF ABBREVIATIONS xix

CHAPTER

1 INTRODUCTION 1.1

2 LITERATURE REVIEW 2.1
2.1 Probiotics 2.1
2.2 Bifidobacteria 2.4
2.2.1 The Organism 2.4
2.2.2 Nomenclature and Classification 2.4
2.2.3 Biology of the Bifidobacteria 2.6
2.2.3.1 Morphology 2.6
2.2.3.2 Cell Wall Structure 2.8
2.2.3.3 Physiology of Bifidobacteria 2.9
2.2.4 Carbohydrate Fermentation 2.11
2.2.5 Factors Affecting F6PPK Activity 2.14
2.2.5.1 Effect of pH 2.14
2.2.5.2 Effects of Temperature 2.14
2.2.5.3 Thermal Stability of F6PPK 2.15
2.2.6 Determination of Acetyl Phosphate 2.15
2.3 Bifidobacteria as A Probiotic Microorganism 2.16
2.3.1 Therapeutic Effects 2.16
2.3.2 Maintenance of Normal Intestinal Microflora Balance 2.17
2.3.3 Alleviation of Lactose Intolerance 2.20
2.3.4 Anti-carcinogenic Activity of Bifidobacteria 2.21
2.3.5 Reduction of Serum Cholesterol Levels 2.22
2.3.6 Synthesis of B-complex Vitamins and Absorption of Calcium 2.24
2.3.7 Immudulation 2.25
2.4 Identification of Bifidobacteria 2.25
2.4.1 Identification Through Morphological Studies 2.27
2.4.2 Identification Through Physiology and Biochemical Characters 2.28
2.4.3 Identification Through Genotypic Studies 2.30
2.5 Immunoassays 2.32
2.5.1 Types of Immunoassays 2.34
2.5.2 Classification of Immunoassays 2.34
2.5.3 Choosing the Correct Design of An Immunoassays 2.37
2.5.4 Types of Detecting System in An Immunoassays 2.37
2.5.5 Principles of Enzyme-Linked Immunosorbent Assay (ELISA) 2.39
2.5.6 Potential uses of ELISA in Routine Food Analysis 2.39
2.5.7 Advantages of Immunoassays 2.40

2.6 Fructose-6-phosphophosphoketolase 2.42

3 MATERIALS and METHODS 3.1
3.1 Bacterial Strains and Culture Condition 3.1
3.2 Bacterial Medium 3.3
3.3 Preparation of Freeze-Dried Cell (B. breve) 3.3
3.4 Gram Staining 3.4
3.5 Extraction of F6PPK (Preparation of Cell Extract) 3.4
3.5.1 Enzymatic Treatment and Bead Milling 3.5
3.5.2 Enzymatic Treatment and Stirring 3.6
3.5.3 Mechanical Disruption: Vortex Homogenation 3.6
3.5.4 Mechanical Disruption: Sonication 3.7
3.5.5 Grinding 3.7
3.6 Purification of F6PPK 3.8
3.7 Gel Electrophoresis 3.10
3.7.1 Native PAGE 3.10
3.8 Measurement of Enzyme Activity and Protein Concentration 3.10
3.8.1 Fructose-6-phosphate Phosphoketolase Assay 3.10
3.8.2 Determination of Protein Concentration 3.12
3.9 Characterization of Fructose-6-phosphate Phosphoketolase 3.12
3.9.1 Effect of Temperature 3.13
3.9.2 Effect of pH 3.13
3.9.3 Thermal Stability 3.13
3.9.4 Storage Stability 3.13
3.9.5 Substrate Specificity 3.14
3.9.6 Effect of Metal Ions 3.14
3.9.7 Effect of Thiol Inhibitors 3.14
3.9.8 Effects of Various Compounds 3.15
3.9.9 Photooxidation of Methylene Blue 3.15
3.9.10 Determination of Kinetic Parameters 3.15
3.9.11 Molecular Mass Determination 3.16
3.10 Production of Polyclonal Antibodies 3.16
3.10.1 Preparation of Immunogen 3.16
3.10.2 Immunization 3.17
3.10.3 Bleeding 3.18
3.10.4 Processing of Serum 3.18
3.10.5 Purification of Antiserum 3.19
3.11 ELISA Tests 3.20
3.11.1 Noncompetitive Indirect ELISA Procedure 3.20
3.11.2 Assessment Of Antiserum Titre for Different Bleeding
3.11.3 Checkerboard Titration 3.22
3.11.4 Determination of Suitable Coating Buffer 3.22
3.11.5 Determination of Suitable Coating Antigen 3.23
3.12.1 Preparation of Samples for Analysis 3.24
3.12.1.1 Extraction of Intracellular Enzyme from Bacteria 3.23
3.12.1.2 Preparation of Bacterial Culture 3.24
3.12.2 Determination of the Specificity and Cross-Reactivity of Anti-F6PPK Antiserum and Anti-B. breve Antiserum

4 RESULTS AND DISCUSSION 4.1
4.1 Effect of Extraction Methods 4.1
4.2 Purification of F6PPK 4.3
4.3 Determination of the Purity of F6PPK By Native PAGE 4.7
4.4 Determination of Molecular Mass of F6PPK 4.8
4.5 Biochemical Characterization of the F6PPK 4.10
4.5.1 Optimum Temperature 4.10
4.5.2 Optimum pH 4.10
4.5.3 Thermal Stability 4.13
4.5.4 Storage Stability 4.13
4.5.5 Substrate Specificity 4.16
4.5.6 Effect of Metal Ions 4.16
4.5.7 Effect of Thiol Inhibitors 4.19
4.5.8 Effect of Protease Inhibitor 4.20
4.5.9 Effect of Various Other Compounds 4.21
4.5.10 Photoxidation of Methylene Blue 4.22
4.5.11 K_m Value 4.23
4.6 Analysis of Antiserum 4.23
4.7 Assessment of Antiserum 4.31
4.7.1 Determination of Antiserum Titre and Optimal Secondary Antibody Concentration 4.31
4.7.2 Determination the Suitability of Coating Buffer 4.38
4.7.3 Determination of Optimal Concentration and Suitability of Coating Antigen 4.41
4.8 Relationship between the Bacterial Count and Absorbance Value 4.45
4.9 Cross Reactivity and Specificity of Antiserum R1 4.49
4.10 Cross Reactivity and Specificity of Antiserum R4 4.57

5 CONCLUSION 5.1

REFERENCES R.1
APPENDICES A.1
Appendix I Working Solutions for Native PAGE A.1
Appendix II Buffer Solutions Used in Indirect ELISA A.2
VITA V.1
RECOMENDATION
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Examples of microorganisms used in probiotic products</td>
<td>2.3</td>
</tr>
<tr>
<td>2.</td>
<td><em>Bifidobacterium</em> species</td>
<td>2.6</td>
</tr>
<tr>
<td>3.</td>
<td>The cell wall composition of different species of <em>Bifidobacterium</em></td>
<td>2.9</td>
</tr>
<tr>
<td>4.</td>
<td>Choosing an assay protocol</td>
<td>2.38</td>
</tr>
<tr>
<td>5.</td>
<td>Relevance of immunoassay to routine food analysis</td>
<td>2.40</td>
</tr>
<tr>
<td>6.</td>
<td>Strains and species of <em>Bifidobacterium</em>, lactic acid bacteria, probiotics, yogurt starter cultures and <em>E. coli</em></td>
<td>3.2</td>
</tr>
<tr>
<td>7.</td>
<td>Enzyme activity, total protein and specific enzyme activity for the crude extract of <em>B. breve</em> (ATCC 15698) by different extraction methods</td>
<td>4.2</td>
</tr>
<tr>
<td>8.</td>
<td>The purification of fructose-6-phosphate-phosphoketolase from <em>B. breve</em> (ATCC 15698)</td>
<td>4.4</td>
</tr>
<tr>
<td>9.</td>
<td>Storage of F6PPK enzyme (crude) with protease inhibitors and antibacterial agents</td>
<td>4.15</td>
</tr>
<tr>
<td>10.</td>
<td>Effects of metal ions on F6PPK activity</td>
<td>4.18</td>
</tr>
<tr>
<td>11.</td>
<td>Effects of thiol inhibitors on F6PPK activity</td>
<td>4.19</td>
</tr>
<tr>
<td>12.</td>
<td>Effects of protease inhibitors (EDTA and PMSF) towards F6PPK activity</td>
<td>4.20</td>
</tr>
<tr>
<td>13.</td>
<td>Effects of various compound towards F6PPK activity</td>
<td>4.21</td>
</tr>
<tr>
<td>14.</td>
<td>Photooxidation of methylene blue towards F6PPK</td>
<td>4.23</td>
</tr>
<tr>
<td>15A.</td>
<td>Study of the cross reactivity and specificity of anti-F6PPK (R1) and anti-<em>B. breve</em> (R4) antiserum against <em>Bifidobacterium</em> spp., probiotics and <em>E. coli.</em></td>
<td>4.65</td>
</tr>
<tr>
<td>15B.</td>
<td>Study of the cross reactivity and specificity of anti-F6PPK (R1) and anti-<em>B. breve</em> (R4) antiserum against <em>Lactobacillus</em> spp., <em>Lactococcus</em> spp, and yogurt starter cultures.</td>
<td>4.66</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Simplified scheme of the metabolism of glucose by bifidobacteria</td>
<td>2.12</td>
</tr>
<tr>
<td>2</td>
<td>Different types of ELISA</td>
<td>2.36</td>
</tr>
<tr>
<td>3</td>
<td>The flat-bottomed 96 wells ELISA plate</td>
<td>3.22</td>
</tr>
<tr>
<td>4</td>
<td>Chromatogram of anion exchanger</td>
<td>4.5</td>
</tr>
<tr>
<td>5</td>
<td>Chromatogram of gel filtration chromatography</td>
<td>4.6</td>
</tr>
<tr>
<td>6</td>
<td>Determination of the purity of F6PPK by native PAGE</td>
<td>4.7</td>
</tr>
<tr>
<td>7</td>
<td>Estimation molecular mass of F6PPK by gel filtration chromatography</td>
<td>4.9</td>
</tr>
<tr>
<td>8</td>
<td>Effect of temperature on F6PPK</td>
<td>4.11</td>
</tr>
<tr>
<td>9</td>
<td>Effect of pH on F6PPK</td>
<td>4.12</td>
</tr>
<tr>
<td>10</td>
<td>Thermal stability of F6PPK</td>
<td>4.14</td>
</tr>
<tr>
<td>11</td>
<td>Substrate specificity of F6PPK</td>
<td>4.17</td>
</tr>
<tr>
<td>12</td>
<td>Lineweaver-Burk plots of F6PPK</td>
<td>4.24</td>
</tr>
<tr>
<td>13A</td>
<td>Assessment of Antiserum Rabbit 1</td>
<td>4.26</td>
</tr>
<tr>
<td>13B</td>
<td>Assessment of Antiserum Rabbit 2</td>
<td>4.27</td>
</tr>
<tr>
<td>13C</td>
<td>Assessment of Antiserum Rabbit 3</td>
<td>4.28</td>
</tr>
<tr>
<td>13D</td>
<td>Assessment of Antiserum Rabbit 4</td>
<td>4.29</td>
</tr>
<tr>
<td>13E</td>
<td>Assessment of Antiserum Rabbit 5</td>
<td>4.30</td>
</tr>
<tr>
<td>14A</td>
<td>Determination of antiserum titre and optimal conjugate antibody of Rabbit 1</td>
<td>4.33</td>
</tr>
<tr>
<td></td>
<td>by checkerboard titration.</td>
<td></td>
</tr>
<tr>
<td>14B</td>
<td>Determination of antiserum titre and optimal conjugate antibody of Rabbit 2</td>
<td>4.34</td>
</tr>
<tr>
<td></td>
<td>by checkerboard titration.</td>
<td></td>
</tr>
<tr>
<td>14C</td>
<td>Determination of antiserum titre and optimal conjugate antibody of Rabbit 3</td>
<td>4.35</td>
</tr>
<tr>
<td></td>
<td>by checkerboard titration.</td>
<td></td>
</tr>
</tbody>
</table>
14D. Determination of antiserum titre and optimal conjugate antibody of Rabbit 4 by checkerboard titration.

14E. Determination of antiserum titre and optimal conjugate antibody of Rabbit 5 by checkerboard titration.

15. Determination of coating buffer absorption towards coating antigen F6PPK and *B. breve*

16A. Determination of the optimal concentration and type of coating antigen for anti-F6PPK antiserum (R1)

16B. Determination of the optimal concentration and type of coating antigen for anti-F6PPK antiserum (R2)

16C. Determination of the optimal concentration and type of coating antigen for anti-F6PPK antiserum (R3)

17A. Relationship between *B. breve* (ATCC 15698)'s plate count and the titre value obtained by indirect ELISA for antiserum R1

17B. Relationship between *B. breve* (ATCC 15698)'s plate count and the titre value obtained by indirect ELISA for antiserum R4

18A. Determination of cross-reactivity and specificity for anti-F6PPK antiserum towards *Bifidobacterium* from ATCC

18B. Determination of cross-reactivity and specificity for anti-F6PPK antiserum towards commercially available *Bifidobacterium*

18C. Determination of cross-reactivity and specificity for anti-F6PPK antiserum towards strains of *B. infantis*

18D. Determination of cross-reactivity and specificity for anti-F6PPK antiserum towards *Lactobacillus spp.* and *L. plantarium*

18E. Determination of cross-reactivity and specificity for anti-F6PPK antiserum towards *Lactococcus spp.*

18F. Determination of cross-reactivity and specificity for anti-F6PPK antiserum towards probiotics, yogurt starter culture and *E. coli*

19A. Determination of cross-reactivity and specificity for anti-*B. breve* antiserum towards *Bifidobacterium* from ATCC

19B. Determination of cross-reactivity and specificity for anti-*B. breve* antiserum towards commercially available *Bifidobacterium*
19C. Determination of cross-reactivity and specificity for anti-\textit{B. breve} antiserum towards strains of \textit{B. infantis} 4.60

19D. Determination of cross-reactivity and specificity for anti-\textit{B. breve} antiserum towards \textit{Lactobacillus spp.} and \textit{L. plantarium}. 4.61

19E. Determination of cross-reactivity and specificity for anti-\textit{B. breve} antiserum towards \textit{Lactococcus spp.} 4.62

19F. Determination of cross-reactivity and specificity for anti-\textit{B. breve} antiserum towards probiotics and yogurt starter culture and \textit{E. coli} 4.63
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>F6PPK</td>
<td>Fructose-6-phosphate phosphoketolase</td>
</tr>
<tr>
<td>TPY</td>
<td>Trypticase-phytone-yeast</td>
</tr>
<tr>
<td>spp.</td>
<td>species</td>
</tr>
<tr>
<td>%</td>
<td>percentage</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>rpm</td>
<td>rotation per minute</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast Protein Liquid Chromatography</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine Tetraacetic Acid</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenyl Methyl Sulfonyl Fluoride</td>
</tr>
<tr>
<td>Native PAGE</td>
<td>Native polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbant Assay</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>FCA</td>
<td>Freund's Complete Adjuvant</td>
</tr>
<tr>
<td>FIA</td>
<td>Freund's Incomplete Adjuvant</td>
</tr>
<tr>
<td>CFU/ml</td>
<td>Colony Forming Unit per milliliter</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>PI</td>
<td>Isoelectric Focusing Point</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
</tbody>
</table>
1.0 INTRODUCTION

The health benefits associated with probiotic organisms have made the food industry incorporate the organisms in many fermented foods and dairy products. At the same time, the health consciousness of consumers has led to an expanding market for foods containing biocultures. One of the species currently employed in probiotic preparations is bifidobacteria.

Bifidobacteria are non-motile, non-spore-forming, gram-positive anaerobic rods of variable appearance. They are natural inhabitants of the gut of warm-blooded animals and human (Mitsuoka, 1982). Bifidobacteria are the major components of intestinal flora of healthy humans as these organisms represent 92% of the fecal flora in breast-fed human (Hori, 1983). These bacteria are considered beneficial for all age groups because they promote resistance to infection in the host (Homma, 1988).

Bifidobacterium can be isolated from different sources such as the feces or guts of human and animals, the human vagina and dental caries (Biavati et al., 1992). These organisms are known as probiotics as they restrict the growth of many potential pathogens and putrefactive bacteria (Rasic, 1989). Bifidobacteria are also believed to have anticarcinogenic (Mitsuoka, 1982; Fernandes and Shahani, 1990) and anticholesterolemic (Hata et al., 1982; Homma, 1988) properties. In addition, bifidobacteria are believed to improve lactose-intolerance and digestibility of milk products (Savaino and Levitt, 1985; Gilliland, 1989). It is ascribed to the synthesis of B-complex vitamins and absorption of calcium (Rasic and Kurmann, 1983; Rasic, 1989). Bifidobacteria have not been associated with infection, in fact it offers a panel of advantages for human health. They are being widely used in dairy products such
as yogurt and bifidus milks. In addition, there is considerable interest in using fermented milks containing bifidobacteria for probiotics purposes (Collins and Hall, 1984).

The genus bifidobacteria includes 32 species, of which 11 are of human origin (Tannock, 1999). At present, there are only 5 available for commercial applications (Tamime et al., 1995). The most suitable species of human origin used for the production of fermented dairy products are *B. longum*, *B. breve*, *B. bifidum* and *B. infantis*. The species *B. animalis* previously isolated from warm-blooded animals have also been found in fermented dairy products (Bonoparte and Reuter, 1991; Biavati et al., 1992).

The increasing number of commercial strains of bifidobacteria used in the food industry requires reliable methods for characterization and control, since it must be ensured that they are of human origin (Biavati et al., 1992). Besides that, as far as human food and animal feed are concern, identifying the origin and strains of the organisms is of paramount importance because of issues to be addressed in relation to regulatory requirements.

Identification of bifidobacteria isolated from stools, commercial dairy products and other materials may be tentative or definitive. So far, *Bifidobacterium* spp. can be distinguished either among species or strains using several techniques. To date, there are several methods that have been proposed for the identification of dairy-related-bifidobacteria. Differentiation of *Bifidobacterium* spp. has mainly been performed on the basis of phenotypic characteristics such as carbohydrate
fermentation patterns and cellular morphology (Scardovi, 1986), or genotypic character-istic, and molecular approach based on DNA-DNA hybridization (Scardovi et al., 1971a). In addition, Bourget et al (1993) has developed a method of identification using specific-specific oligosaccharide probes. Recently, specific detection of bifidobacterium strain in infant feces by gene rRNA primer probes has been developed (Kok et al., 1996).

The definitive identification of a bifidobacteria strain may be accomplished with procedures such as DNA/DNA hybridization (Scardovi et al., 1971a) or electrophoretic protein patterns (Bia-vati et al., 1982). However, these methods are difficult to perform routinely in a food laboratory. DNA-DNA hybridization studies have also demonstrated that the levels of DNA relatedness between these two species (B. breve and B. infantis) are very similar (Lauer and Kandler, 1983; Bahaka et al., 1993). Yamamoto et al (1992) also observed the oligonucleotide probes for bifidobacteria of human origin cross-reacted with a few strains of heterologous Bifidobacterium species of non-human origin.

The elucidation of the unusual means of hexose metabolism in the bifidobacteria studied by Scardovi (1981) revealed that the enzyme, fructose-6-phosphate phosphoketolase (F6PPK), to be unique among lactose utilizing bacteria. Assay for the activity of this enzyme has become a key-differentiating test for the identification of bifidobacteria. Bifidobacteria can be distinguished from other bacterial group like lactobacilli, actinomyceae and anaerobic corynebacteria by the peculiar pathway: “fructose-6-phosphate shunt” (Scardovi, 1986) leading to the formation of lactic acid and acetic acids in the ratio 1.0:1.5 as chief end products.
However, in the case of bifidobacteria, analysis of volatile or metabolite products is of limited value since lactic and acetic acids are the main end products for all species. Scardovi et al. (1971b) have studied the F6PPK electrophoretic mobility and demonstrated that this enzyme shows different patterns according to bifidobacteria ecology. However, *B. globusum* and *B. dentium* were shown to possess an “animal” and “human” electrophoretic type of F6PPK, respectively (Grill et al., 1995).

Among the various methods mentioned above, nonimmunological methods are not convenient for routine sample analyses since they are relatively costly and time-consuming and require technical expertise (Levieux and Venien, 1994). Moreover, a large data bank of various species is needed for effective protein profile comparison and DNA-DNA hybridization pattern. Immunochemical techniques provide complementary and alternative approaches to reduce the use of costly, sophisticated equipment and analysis times, while still maintaining reliability and improved sensitivity (Rosalba et al., 1997).

Immunochemical techniques are potentially useful for the routine microbiological analysis of foods due to its specific nature and high sensitivity of the antibody-antigen reaction (Candlish, 1991). The interest of the immunological methods lies in their advantages of sensitivity, specificity, and cost-effectiveness. Other advantages include the simplicity of the procedures for sample preparation and the capability of routine and simultaneous analysis of large numbers of samples. The use of immunoassays by the food industry is increasing steadily. Among these Enzyme-Linked Immunosorbent Assay (ELISA) being the most widely used immunoassay in food analysis (Allen, 1990). Applications of immunological assays