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Amplification and sequence analysis of TPI gene from Lactobacillus delbrueckii and its Antimicrobial activity testing

Deshpande Archana
deshpandearchana88@gmail.com
M.G.R COLLEGE OF ARTS & SCIENCE , Tamil Nadu

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Abstract
The last decade has seen much renewed interest in the area of bacteriocin. In particular, those that derived from Lactobacillus bacteria also called Lactic acid bacteria (LAB).LAB is Gram-positive, facultative anaerobic, non motile and non spore forming, rod shaped bacteria. Which are both agriculturally and industrially important. The preservative ability of LAB in foods is attributed to the production of anti-microbial metabolites including organic acids and bacteriocins which are the predominant components of probiotics. Isomerses are known to convert the alternative forms of a substrate into vice versa. TPI Triose Phosphate isomerase is one such enzymes produced by LAB and aids in the inter conversion of the two isomeric forms dihydroxyacetone phosphate and D-glyceraldehyde3-phosphate. The enzyme plays a vital role in glycolysis and ATP generation. It is ubiquitously occurring in huge species like Humans, insects, bacteria, fungi etc. In humans deficiency of this gene occurs as a result of autosomal resessive gene inheritance causing a type of rare glycolytic enzymopathy. The condition may lead to several clinical consequences which include chronic haemolytic anemia, cardiomyopathy, susceptibility to infections, severe neurological dysfunction and in most cases, death in early childhood.

Understanding the importance of this gene and its regulation in the metabolic activities, the detailed genetic and protein analysis of TPI is aimed in the present work. As the gene is commonly found in Lactobacillus bacteria, it has been targeted for the isolation of Genetic material and amplification of TPI gene followed by its annotation. Bacteria required for gene extraction is isolated from curd samples and subjected for pure culturing followed by identification. The identified pure culture is used for genomic DNA extraction and amplification of TPI gene followed by sequencing and annotation. Several Biotechnological and Bioinformatics techniques have been used in the study which are elaborated.

Keywords: Triose phosphate Isomerase, Glycolysis, Isomers, Lactic Acid Bacteria, Autosomal resessive inheritance.

Introduction
Triose phosphate isomerases are one of the house keeping genes of majority of the higher organisms involved in vital cellular metabolisms. The gene is located on the 12th chromosome on the P arm at the position 13 represented as 12P13 [11]. They are a group of isomerases involved in the conversion of a 3 carbon compound dihydroxyacetone phosphate to D-glyceraldehyde3-phosphate in the initial steps of glycolysis. In most of the organisms its functional state is an oligomer in the form of homo dimer [8].
Alterations associated with gene results in several abnormalities which are not limited to humans but seen in all organisms. Further gene is also reported to play a predominant role in cancers. The elevated expression of this gene is reported in several tumors [10]. It is also known to have a key role in Gastric cancer which was studied by several researchers. Apart from the disturbances due to TPI elevation , there are several diseases associated with the deficiency of TPI. TPI deficiency is one of the glycolytic enzyme defects and causes progressive neurological dis function and is associated with frequent childhood death [9]. There are several mutations observed in this gene and these mutations are associated with the deficiency of TPI. Once such well studied mutation is Glu104Asp [4]. Lactc acid bacteria is an organism that also possess this gene, further the role of it in Operon is quite essential. Lactic acid bacteria is used in the present study for the isolation of this gene and its characterization.

Material and Methods
Lactic acid Bacteria, one of the basic organism which needs no special requirements for culture and exhibits good growth rate is used in the work. Lactic acid bacteria was aimed to be isolated from curd followed by its use in the extraction of genomic DNA for targeting the sequencing of TPI gene for

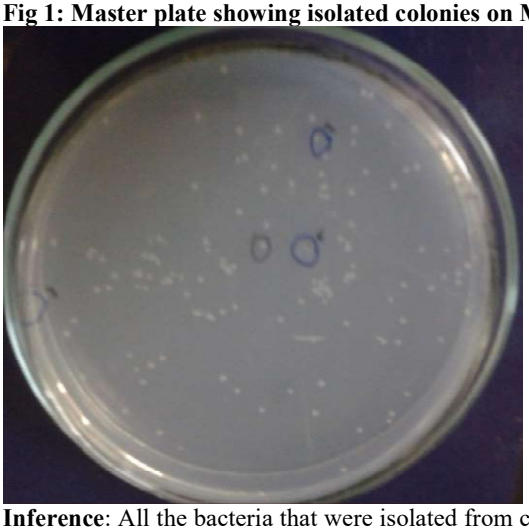
further analysis. The procedure following extraction was to target the TPI gene amplification, for which primers were screened and designed.
Initial isolation of bacteria was performed on MRS [3] media using diluted curd as an inoculate for spreading. Plates were incubated for standard time of 24 hrs overnight to obtain isolated colonies.
Master plate containing isolated colonies was observed and used further in perpetration of pure cultures. Colonies were selected based on colony morphology and identification is expected to be performed after pure culture growth. All the pure cultures were subjected for gram's staining [2] followed by identification based on biochemical tests. Based on the results only the bacteria *Lactobacillus delbrueckii* was selected for further study [6]. Culture was used for broth preparation to obtain a uniform cell suspension. Genomic DNA was extracted from suspension using standard protocol based on phenol chloroform method [13].
Extraction was followed by purification based on ethanol wash. Primers were designed and used for amplification in PCR. 35 cycles of PCR were run with the prescribed standard conditions. Amplicons were then subjected for sequencing using automated ABI sequencer 3500 [1] which runs on the principle of Sanger sequencing and capillary electrophoresis. The sequence thus received was further used for analysis using various insilico methods.

Insilico gene sequence analysis: Once the PCR amplicons were obtained the samples were subjected for Sequencing and the sequence obtained was used for insilico gene analysis. The sequence was first subjected for BLAST N [5] to check its identity followed by Molecular weight determination, Codon plot and Translate tool to obtain the protein product [12]. The protein sequence thus obtained can further be subjected for BLAST P for final conformation of identity. 3D structure of the protein was obtained using PHYRE tool [7].

Results and Discussion

Initially the curd samples were diluted and used for inoculating the MRS media plates using spread plate method to obtain the colonies of pure culture. 4 colonies were selected for pure culture followed by identification. Two of the 4 were identified to be *Lactobcillus delbrueckii* whose results are furnished below.

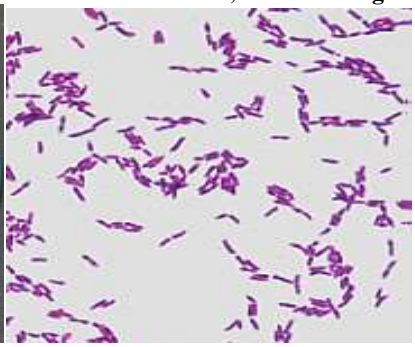
Fig 1: Master plate showing isolated colonies on MRS media plates:



Inference: All the bacteria that were isolated from curd for the 1st time onto the MRS media are shown in fig 1

Pure culture preparation from the above plates can be performed. The plate is shown below:

Fig 2, 3: The Pure culture Plate of one of the colonies streaked, 3: Result of gram staining



Inference: The pure culture streak shows the colony morphology to be white, creamy texture with slimy appearance. Colonies were further subjected for staining and the results are shown in fig 3. Bacteria were identified to be gram positive bacilli.

Biochemical tests can be performed for further identification.

Table 1: Results of Biochemical tests

S.No	Biochemical Test	Result
1	Catalase Test	Negetive
2	Citrate est	Positive
3	Indole Test	Negetive
4	Methyl red Test	Positive
5	VP Test	Negetive
6	Starch Test	Positive

Inference: The above biochemical tests and their results upon analysis show that the Bacteria isolated was *Lactobcillus delbrueckii*

Gene sequence retreival of TPI *Lactobcillus delbrueckii* from NCBI

tpiA triose-phosphate isomerase [Lactobacillus delbrueckii subsp. jakobsenii ZN7a-9 = DSM 26046]

>NZ CP018218.1:906752-907510 Lactobacillus delbrueckii subsp. jakobsenii ZN7a-9 = DSM 26046 chromosome, complete genome

ATGTCACGTACCCCAATTATTGCTGGTAACTGGAAGCTGAACATGAACCCAAAGGAAACTGTTGAGTTCG
TAAACGCTGTTAAGGACCAATTGCCAGACCCATCAAAGGTTGAATCGGTGATTTGTGCACCAGCAGTTGA
CTTGGACGCCTTGCTGAAGGCTGCCGAAGGCTCAAACCTGCACGTCGGTGCAGAAAACTGCTACTGGGAA
AACTCCGGTGCCTTCACTGGTGAAACTTCTCCAGCAGTTTTGAAGGAAATGGGCGTGCAATATGTCATCA
TCGGACACTCAGAACGCCGTGACTACTTCCACGAAACTGACGAAGACATCAACAAGAAGGCCAAGGCTAT
CTTTGCCAACGGCTTGACTCCAATCCTTTGCTGCGGTGAATCACTGGAAATCCGGGAAGCCGGCAAGGAA
AAAGAATGGGTTGTCAGCCAAATCAAGGCTGACCTGGAAGGCTTGACTAGTGAACAAGTTTCCAAGCTGG
TGATCGCTTACGAACCAATCTGGGCGATTGGCACTGGCAAGACTGCTTCAAGCGACCAAGCTGAAGAAAT
GTGCAAGACTATCCGGGAAACTGTTAAGGACTTGTACAACGAAGAAACTGCTGAAAACGTCCGCATCCAA
TACGGCGGTTCAGTAAAGCCAGCCAACATCAAGGAATTGATGGCTAAGCCAAACATCGACGGTGGCTTGG
TTGGTGGTGCATCATTGGTACCAGACTCATACCTGGCTTTGGTAAACTACCAAGACTAA

Inference: TPI coding gene sequence of selected organism was 759bp long and falling in the region 906752 - 907510.

Primer designing for PCR amplification :

Primer 3 tool was used for obtaining the potential primers which can be used for amplification and sequencing. The primers proposed by PRIMER 3 are shown below

Fig 4: Results of Primer 3

```
Template masking not selected
No mispriming library specified
Using 1-based sequence positions
OLIGO           start  len     tm     gc%  any th  3' th hairpin seq
LEFT PRIMER        85   20   58.89   55.00    0.00   0.00    0.00 GACCAATTGCCAGACCCATC
RIGHT PRIMER      308   20   59.04   50.00   11.19   1.14    0.00 AAGTAGTCACGGCGTTCTGA
SEQUENCE SIZE: 759
INCLUDED REGION SIZE: 759

PRODUCT SIZE: 224, PAIR ANY_TH COMPL: 0.00, PAIR 3'_TH COMPL: 0.00
```

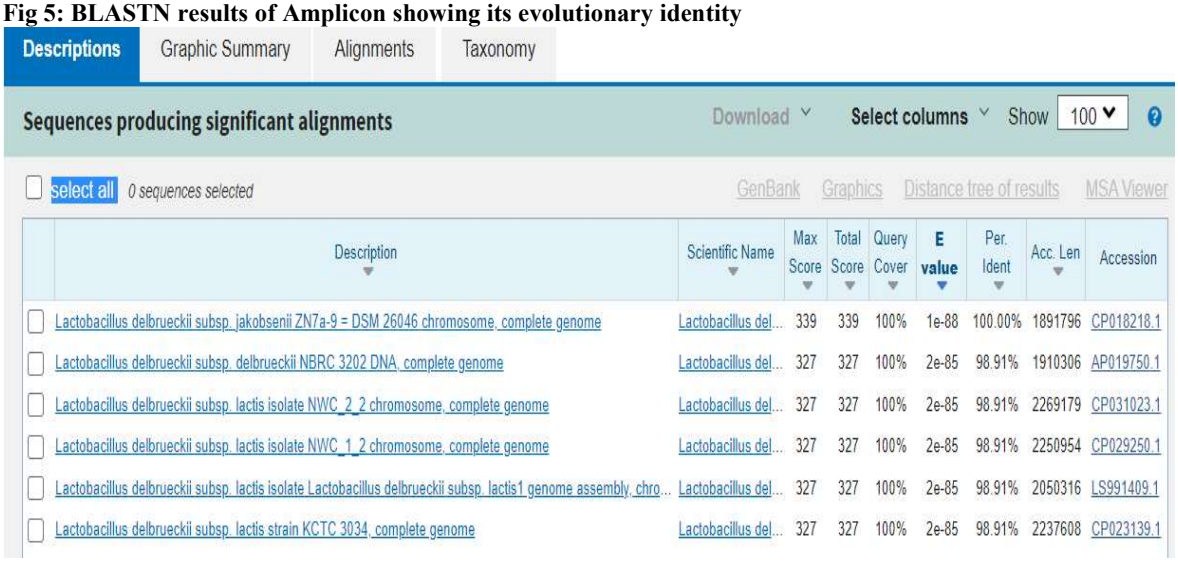
Inference: Based on the above results the set of primers selected for amplification of TPI gene from Lactobacillus are Forward Primer: GACCAATTGCCAGACCCATC and Reverse primer: AAGTAGTCACGGCGTTCTGA. The amplicon size expected from this set of primers is 224bp.

Results of ABI 3500 sequencing:
Sequencing results yielded the DNA fragment with the sequence shown below

AAAGGTTGAATCGGTGATTTGTGCACCAGCAGTTGACTTGGACGCCTTGCTGAAGGCTGCCGAAGGCTC
AAACCTGCACGTCGGTGCAGAAAACTGCTACTGGGAAAACTCCGGTGCCTTCACTGGTGAAACTTCTCC
AGCAGTTTTGAAGGAAATGGGCGTGCAATATGTCATCATCGGACAC

The above sequence is subjected for BLASTN analysis for identification of its evolutionary origin

Fig 5: BLASTN results of Amplicon showing its evolutionary identity



Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Lactobacillus delbrueckii subsp. jakobsenii ZN7a-9 = DSM 26046 chromosome, complete genome	Lactobacillus del...	339	339	100%	1e-88	100.00%	1891796	CP018218.1
Lactobacillus delbrueckii subsp. delbrueckii NBRC 3202 DNA, complete genome	Lactobacillus del...	327	327	100%	2e-85	98.91%	1910306	AP019750.1
Lactobacillus delbrueckii subsp. lactis isolate NWC_2_2 chromosome, complete genome	Lactobacillus del...	327	327	100%	2e-85	98.91%	2269179	CP031023.1
Lactobacillus delbrueckii subsp. lactis isolate NWC_1_2 chromosome, complete genome	Lactobacillus del...	327	327	100%	2e-85	98.91%	2250954	CP029250.1
Lactobacillus delbrueckii subsp. lactis isolate Lactobacillus delbrueckii subsp. lactis1 genome assembly, chro...	Lactobacillus del...	327	327	100%	2e-85	98.91%	2050316	LS991409.1
Lactobacillus delbrueckii subsp. lactis strain KCTC 3034, complete genome	Lactobacillus del...	327	327	100%	2e-85	98.91%	2237608	CP023139.1

Inference: The above result of BLAST confirm the identity of the sequence to be from *Lactobcillus delbrueckii.*

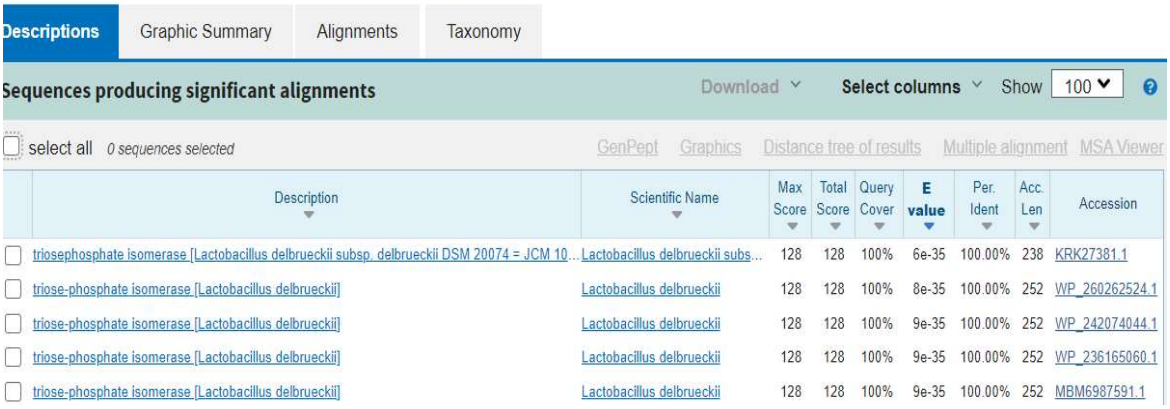
Results of the tool, DNA Molecular weight as calculated from sequence are shown below:
DNA Molecular Weight results
Results for 183 residue sequence "Untitled" starting "AAGGTTGAAT"
56729.72 Da
In order to understand the exact coding pattern CODON PLOT was used whose results are shown below:
Fig 6: CODON PLOT results

Codon Plot results
Results for 183 residue sequence "Untitled" starting "AAGGTTGAAT"

```
aag, 1 to 3 (Lys)
XXXXXXXXXXXXXXXXXXXXXXXXX 0.25

gtt, 4 to 6 (Val)
XXXXXXXXXXXXXXXXXXXXXXXXXX 0.27

gaa, 7 to 9 (Glu)
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX 0.68

tcg, 10 to 12 (Ser)
XXXXXXXXXXXXXX 0.15

gtg, 13 to 15 (Val)
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX 0.36

att, 16 to 18 (Ile)
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX 0.50
```

Translate tool for obtaining the Protein product for gene:
>rf 1 Untitled
KVESVICAPAVDLDALLKAAEGSNLHVGAENCYWENSGAFTGETSPAVLKEMGVQYVIIG H

Fig 7: BLASTP results for the above translated sequence to identify its identity are shown below:

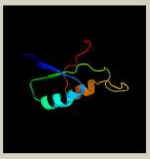


Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
triosephosphate isomerase [Lactobacillus delbrueckii subsp. delbrueckii DSM 20074 = JCM 10...	Lactobacillus delbrueckii subs...	128	128	100%	6e-35	100.00%	238	KRK27381.1
triose-phosphate isomerase [Lactobacillus delbrueckii]	Lactobacillus delbrueckii	128	128	100%	8e-35	100.00%	252	WP_260262524.1
triose-phosphate isomerase [Lactobacillus delbrueckii]	Lactobacillus delbrueckii	128	128	100%	9e-35	100.00%	252	WP_242074044.1
triose-phosphate isomerase [Lactobacillus delbrueckii]	Lactobacillus delbrueckii	128	128	100%	9e-35	100.00%	252	WP_236165060.1
triose-phosphate isomerase [Lactobacillus delbrueckii]	Lactobacillus delbrueckii	128	128	100%	9e-35	100.00%	252	MBM6987591.1

Inference: Based on the above result the final translated protein was conformed to be TPI product of *L. delbreuckii*

PROTPARAM for the above sequence to analyse the basic physicochemical characters:
The results of protparam revealed several characters of protein which include : The molecular weight: 6404.25 Da, with the length of 61 amino acids. Isoelectric point was calculated to be 4.58. Total no of negative amino acids were calculated to be 8 and positive to be 3 indicating that the protein is acidic in nature with its extinction co efficient calculated to be 280nm.

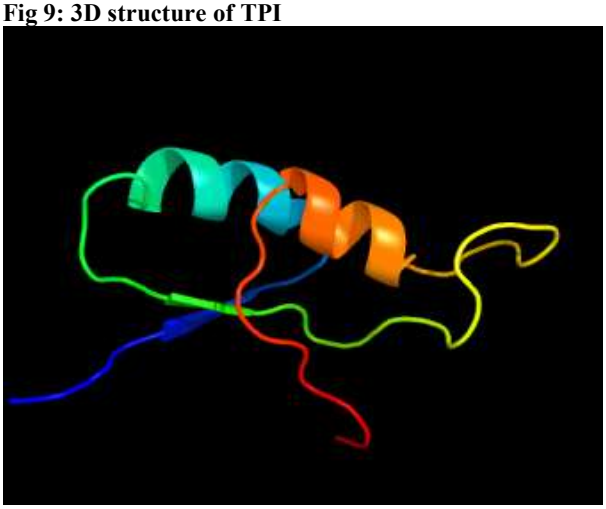
Fig 8: Phyre Result for the above protein sequence:

Template	Alignment Coverage	3D Model	Confidence	% i.d.	Template Information
c5ibxB	Alignment		99.9	66	**PDB header:**isomerase **Chain:** B: **PDB Molecule:**triosephosphate isomerase; **PDBTitle:** 1.65 angstrom crystal structure of triosephosphate isomerase (tim)2 from streptococcus pneumoniae **PDB Entry:** PDBe RCSB PDBj Run Investigator
c3m9yB	Alignment		99.9	62	**PDB header:**isomerase **Chain:** B: **PDB Molecule:**triosephosphate isomerase; **PDBTitle:** crystal structure of triosephosphate isomerase from methicilli resistant staphylococcus aureus at 1.9 angstrom resolution **PDB Entry:** PDBe RCSB PDBj Run Investigator

Inference: Based on the above result of Phyre it can be concluded that the Structure of TPI protein is available in the database, however the protein is from several other organisms like Staphylococcus or homo sapiens but not from Lactobacillus. Thus the structure showing 99.9% confidence and 66% identity with the PDB ID **5ibx** is selected as the close structure for the sequence.

The structure is shown below:

Fig 9: 3D structure of TPI



Inference: 3D structure of the query protein Triose Phosphate Isomerase

Conclusion

With the known importance of Triose posphate isomerase gene, the present study aimed to isolate and analyze the TPI gene of Lactobacillus. Study stared with the initial isolation of bacteria from soil followed by its pure culture preparation and biochemical identification. The bacteria Lactobacillus was subjected or genomic DNA extraction using Phenol chloroform protocol and the purified DNA was amplified using the designed primers. The amplicons were purified and sent for sequencing based on Sanger method. The sequence obtained was further subjected for Gene and translated protein analysis. All the results were aggregated to understand the nature of gene and characters of protein. The protein 3D structure was also obtained from PDB data base.

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