Helix (2022) 12 (4):1-8 https://doi.org/10.29042/2022-12-4-1-8

Amplification, sequence analysis and transformation of TPI gene from Lactobacillus delbrueckii and insilico testing of its anticancerous activity

[1] Ravikanth, [2] Jyothsna Gundlapally
[1, 2] BioAxis DNA Research Centre, Hyderabad
jyothsna@dnares.in

Received: June 2nd 2022, Accepted: 28th June 2022, Published: 31st August 2022

Abstract
Triose phosphate isomerase is one of the bacterial genes that plays a role in basic metabolic processes involving conversion of complex form of chemicals to other simpler forms. This gene codes for an enzyme essential for a bacteria to convert fructose and glucose into simple components and ATP by the cells. Lack of this enzymes leads to an inability of strains to utilize these sugars from the environment. The present work involves the study of transformation of this gene from wild bacterial strains to the mutants that lack the gene. The detailed insilico analysis of the gene was also included in the study. In addition to this the study involves isolation and identification of *Lactobacillus delbrukii* from milk and curd samples aiming at the amplification of their TPI gene. The isolated gene is targeted to be transformed into selected bacteria. The transformation was performed using vector plasmid pAS 100 by the process of conjugation with mutants of *E coli.* These mutants are known for their inability to grow on fructose and glucose due to the lack of Triose phosphate isomerase activity. These trans conjugates recovered were known to possess the ability to grow on fructose and glucose. Major active sites were predicted using insilico tools and docking of TPI with EGFR protein as the cancer receptor was analyzed.

Keywords: TPI gene, Mutants, Amplification, Transformation, Plasmid, pAS 100 plasmid

Introduction
Lactobacillus, which is also commonly known to be Döderlein's bacillus, is a gram-positive bacteria which is facultative anaerobic or microaerophilic rod-shaped bacteria widely distributed in nature [10]. They form the major group of the lactic acid bacteria. They are named as Lactobacillus group due to their ability to convert lactose and other sugars to lactic acid [2]. Apart from natural environment these bacteria are also known to be common inhabitants of human body and are included in the group of friendly bacteria. In humans these bacteria are localized in organs like vagina and the gastrointestinal tract where they constitute the normal gut flora. They are usually benign, except in the mouth where they have been associated with cavities and tooth decay (dental caries) [6]. In order to obtain them as pure culture for the study they can be easily isolated from milk and other dairy products.

Some of the species of *Lactobacillus* are used at industrial scale in the production of dairy products like yoghurt, cheese and other fermented foods like saukraut, pickles, beer, wine, cider, kimchi etc [8]. Sourdough bread is made using a "starter culture", which is a symbiotic culture of yeast and lactic acid bacteria growing in a water and flour medium [1]. *Lactobacilli,* especially *L.casei* and *L.brevis* are some of the most common organisms involved in the spoilage of beer [5].
Among the lactobacillus group *Lactobacillus delbrueckii* is a rod shaped bacteria which is gram positive and a non-motile bacterium with a common sugar fermentation ability producing final product to be lactic acid anaerobically [4]. Except *L. delbrueckii subsp. delbruecki* all the other species are naturally present in milk and dairy products [7]. *L. delbrueckii subsp. delbruecki* is mostly present in vegetable sources.

Triose-phosphate isomerase (TPI or TIM) is one of the protein enzymes which is responsible for the reversible conversion of the triose phosphate isomers dihydroxyacetone phosphate to D-glceraldehyde 3-phosphate [3]. TPI has a vital role in glycolysis and is necessary for the production of energy efficiently. TPI being a universal enzyme in all organisms can be isolated and engineered from any organism. This gene can be used in several therapeutic procedures [9].

Triose phosphate isomerase is a highly efficient enzyme which can perform the reaction several times faster than any other natural processes.

Materials and Methods

Isolation and Identification of *L. delbrueckii* from milk sample

All the standard microbiology protocols were used to isolate the lactobacillus species from milk, which include initial maser plate preparation on nutrient agar media followed by streak plate method on MRS media. The isolated colonies were subjected for gram's staining, microscopy followed by biochemical tests. Pure cultures of *L. delbrueckii* were further inoculated in liquid broth for multiplication and further study.

Genomic DNA extraction from bacterial cells in broth [11]

1ml of overnight bacterial culture was collected in a small graduated centrifuge tube and was spin at 10000 rpm for 10 minutes. The pellet obtained was further mixed with 100ul of T.E buffer(1x) to which 10ul of lysozyme was added. The tubes were incubated at 37^0C for 30 minutes. Later 20ul of 10% SDS and 15ul proteinase k were added to the same tube and re incubated at 60^0C for an hour. To the above tubes 400ul of 6M ammonium acetate was added and votexed thoroughly. The solution was centrifuged at 10000rpm for 6 minutes. The supernatant was collected to which equal volume of chilled isopropanol/ ethanol was added for precipitation of DNA. The sample was incubated at -20^0C for 30 minutes or alternatively incubation can be performed at 40^0C overnight. The tubes were centrifuged at 10000 rpm for 10 minutes. Pellet thus obtained was subjected for 70% ethanol wash followed by air drying. The pellet is again dissolved in 1X T.E buffer until use.

Agarose gel electrophoresis

Before performing the gel run agarose gel electrophoresis tray and chamber were cleaned and wiped with ethanol followed by air dry. The gel tray/ mold was sealed with cellophane tape and tested for leakage. To the tray 50 ml of 0.7% agarose prepared in TAE buffer and dissolved in an oven was added. In order to settle the gel it was cooled to 45^0C and 18µl Ethidiun bromide a DNA staining dye was added. It was mixed and poured into agarose gel tray. The comb was inserted based on the no of samples used for separation and was left for setting. The tray should not be disturbed until complete solidification. After setting, the comb was removed carefully without puncturing the wells. The seal was removed and the gel tray was placed in the electrophoresis chamber. The gel was covered with TAE buffer. Bacterial genomic DNA samples were prepared for loading into the wells as mentioned below :

10µl of DNA + 5µl of loading dye

The bacterial genomic DNA extracted cab be loaded carefully. The power supply can be switched on and gel is allowed to run till the bands reaches 3/4th of the gel at 50V. The gel was observed for visualization of genomic DNA bands in UV transilluminator

Primer designing

One of the prerequisites for Primer designing is the knowledge about target gene sequence. Thus the gene sequence of TPI was retrieved from NCBI public data base, which is redundant and free. While selecting the gene sequence it is important to check that the sequence is 100% homologous to the bacteria which was used for DNA extraction. Additionally it must be cross checked that the sequence specifies only one distinct gene. Primer 3 tool can be used which is available online at https://primer3.ut.ee/. Parameters have to be specified and thus primer can be generated. Some of the important points considered during primer designing include GC content, melting temperature and product length. The primers obtained would be of 20bp length and in pair containing a forward primer and a reverse primer to amplify the target gene region.

Amplification of TPI gene by PCR

For the amplification of TPI gene from the genomic DNA extracted, primers developed as above were used. A pair of primers one forward and other reverse with other inputs which are specified further are added in PCR mix and subjected for amplification. All the components used for PCR amplification are:

Template – 4ul, Forward primer -2ul, Reverse primer-2ul, Taq DNA polymerase -1ul, DNTPs -2ul, MgCl2 – 1ul, PCR Buffer – 5ul, Distilled water-9ul

The programme used in thermocycler is as follows with the specified conditions:

- **Pre PCR Cycle:** Initial denaturation of template DNA at 94^0C for 5 minutes
- **PCR Cycle:** Denaturation for 1min at 94^0C. Primer annealing for 1min at 55^0C . Extension at 72^0C for 1min. Number of cycles to be set for 35.
- **Post PCR Cycle:** Final extension at 72^0C for 5 minutes

Agarose gel electrophoresis for PCR products

Amplified DNA samples were run for electrophoretic analysis on 1.2% agarose gel containing Ethidium bromide and analysis of gel was done under UV light.

Transformation of TPI gene into *E coli* cells

- The amplicons of TPI were transformed into mutated strains of *E coli* that lack the TPI gene. Following steps were involved in this transformation:
- Preparation of recombinant vector (pAS 100)
- Competent cell preparation of *E coli*
- Conjugation of recombinant vector in competent cells
- Screening for TPI expression in *E coli*

Preparation of Recombinant pAS 100 and ligation

Recombinant pAS 100 was prepared by following 2 basic steps. Initial restriction digestion of DNA with EcoRI followed by TPI amplicons ligation of *L delbrukii* with the digested vector. Initially the pAS 100 vector was subjected for restriction digestion and later subjected for ligation. The ligation was carried out as shown below: All the components specified are added to a PCR or Eppendorf tube and are incubated at 37^0C for 1hr (to accelerate enzyme activity). Vector DNA-2.5 µL, Insert DNA-7.5 µL, 10X Ligase Buffer-2.5µL, T4 DNA Ligase-2µL, water to make up the volume to 25µL. These recombinant plasmids were transferred by conjugation to mutants of *E col*i which were unable to grow on fructose and gluconate due to the lack of Triose Phosphate isomerase activity.

Initially *E coli* cells were imparted the competency by chemical treatment and later subjected for transformation.

Procedure for recombination

To the pellet of competent cells 2ul of recombinant plasmid pAS 100 was added carefully. This was followed by flicking the tubes 4-5 times to mix cells and DNA. Vortexing should be avoided. Mixture was placed on ice for 30 minutes followed by heat shock at 42°C for 30 seconds.

The tube is placed on ice for 5 minutes to which 1ml of sterile LB broth was added. This was kept in shaker incubator at 37^0C at 250rpm for 1 hour.

Screening for TPI expression in E coli

50ml of LB agar medium was prepared in which tryptone was substituted with glucose as the carbon source. Incubated culture suspension was inoculated onto solidified LB agar plates.

Transformed colonies were observed after overnight incubation. If colonies were successfully transformed it can be indicated by the utilization of glucose by *E.coli* cells which were initially incapable. This can be tested by their growth on Minimal Salt media with Glucose as carbon source.

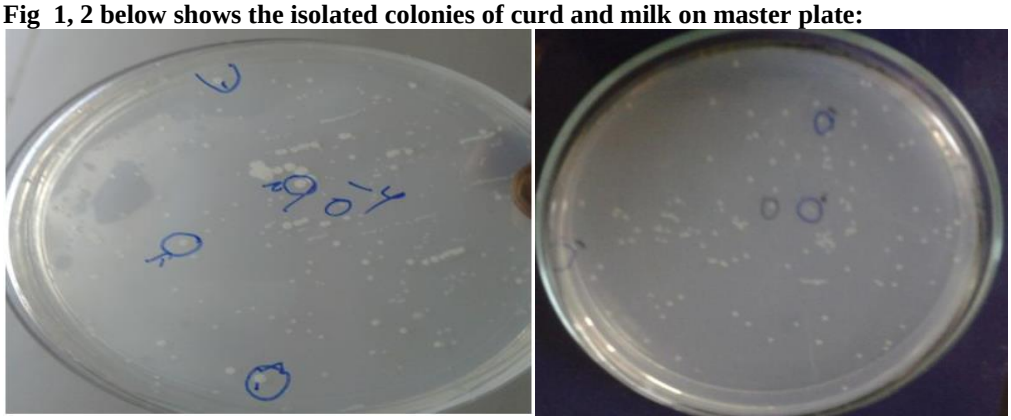
Insilico analysis

This study included the Sequence retrieval of TPI gene from NCBI public data base followed by its sequence and structural study. Apart from its basic structural annotation the study focused on testing its ability against cancer cell proliferation based on docking studies with cancer cell receptor EGFR. The docking studies were performed in MOLEGRO SOFTWARE and 3D structure was visualized in Argus lab.

Results and Discussion

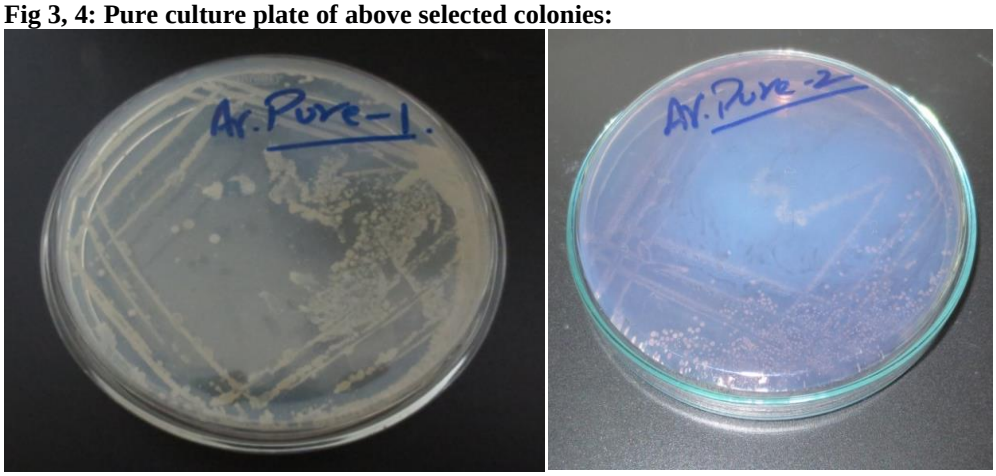
Isolation of Lactobacillus delbrueckii from milk and curd

Fig 1, 2 below shows the isolated colonies of curd and milk on master plate:



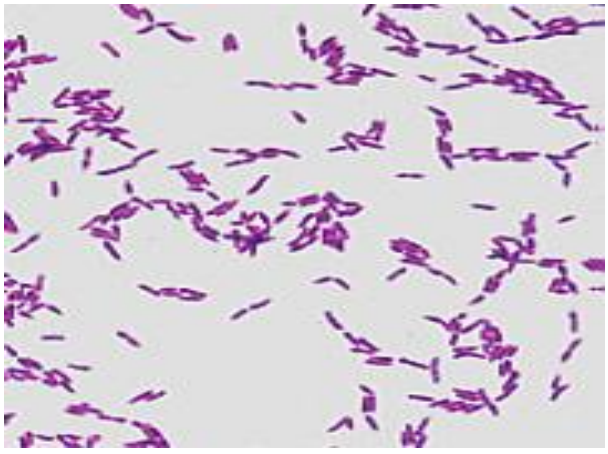
Inference: The above plate shows isolated colonies on NAM and the markings indicating the colonies to be sub cultured.

Fig 3, 4: Pure culture plate of above selected colonies:



Inference: The colony morphology was observed as White coloured, powdery form, circular colonies for milk sample and white coloured, small dot like colonies for curd.

Fig 5: Gram staining of colonies from milk



Inference: Gram positive rods were observed for the above test colonies under 100x oil immersion objective.

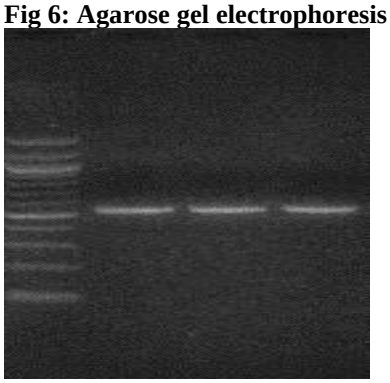
Table 1: Results of biochemical tests performed for the test culture

S.NO	BIOCHEMCIAL TEST	RESULT
1	Gas from Glucose	Negative
2	Acid from Galactose	Positive
3	Acid from Lactose	Positive
4	Acid from Maltose	Positive
5	Acid from Mannitol	Negative
6	Nitrate Reduction	Negative
7	Growth at 45^0C	Positive
8	Indole utilization	Negative
9	Motility	Mostly Negative
	H2S prodction	Negative
11	Catalase	Negative
12	Citrate	Negative

Inference: The above table shows the list of biochemical tests that can be considered as reference for the identification of *Lacobacillus delbreuckii*

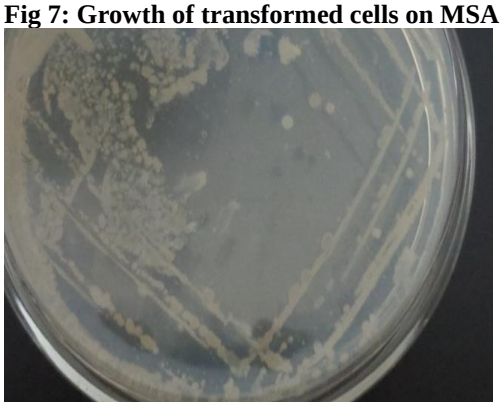
Primers designed for TPI Amplification
Forward primer: GGTAAGCTGCCAGATCCAAG
Reverse primer: ACCGATAGCCCAATTGGTT

Fig 6: Agarose gel electrophoresis with amplicons after PCR amplification:



Inference: The above pictures shows the Amplicons in he 2, 3 and 4th well loaded in replicas and the 1st well showing the ladder.

Fig 7: Growth of transformed cells on MSA media with glucose as Carbon source



Inference: Above picture shows the successful growth of transformed *E coli* cells on MSA with glucose as sole carbon source.

In silico Sequence analysis of TPI gene:

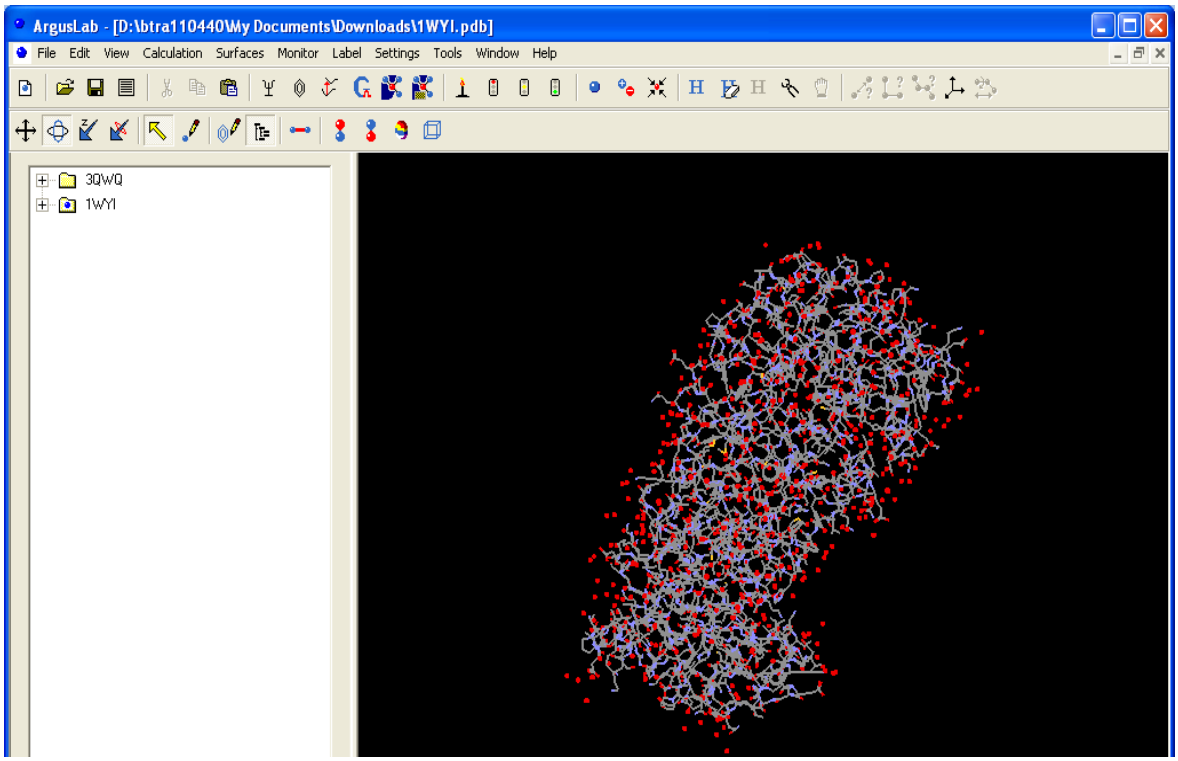
TPI Protein sequence from NCBI

>gi|67473567|sp|P0A858.1|TPIS_ECOLI RecName: Full=Triosephosphate isomerase; Short=TIM; AltName: Full=Triose-phosphate isomerase

MRHPLVMGNWKLNGSRHMVHELVSNLRKELAGVAGCAVAIAPPEMYIDMAKREAEGSHIM
LGAQNVDLNLSGAFTGETSAAMLKDIGAQYIIIGHSERRTYHKESDELIAKKFAVLKEQGLTPV
LCIGETEAENEAGKTEEVCARQIDAVLKTQGAAAFEGAVIAYEPVWAIGTGKSATPAQAQAV
HKFIRDHIAKVDANIAEQVIIQYGGSVNASNAAELFAQPDIDGALVGGASLKADAFAVIVKAA
EAAKQA

Based on the above sequence PDB BLAST and PHYRE tool were used to select the best 3D structure for TPI sequence. The PDB Id selected as structure representative for TPI was **1WYI**. This was further used for docking with the EGFR structure, which was selected as target receptor for studying the inhibitory effect of TPI on cancer.

Fig 8: 3D structure of TPI in 1Argus lab

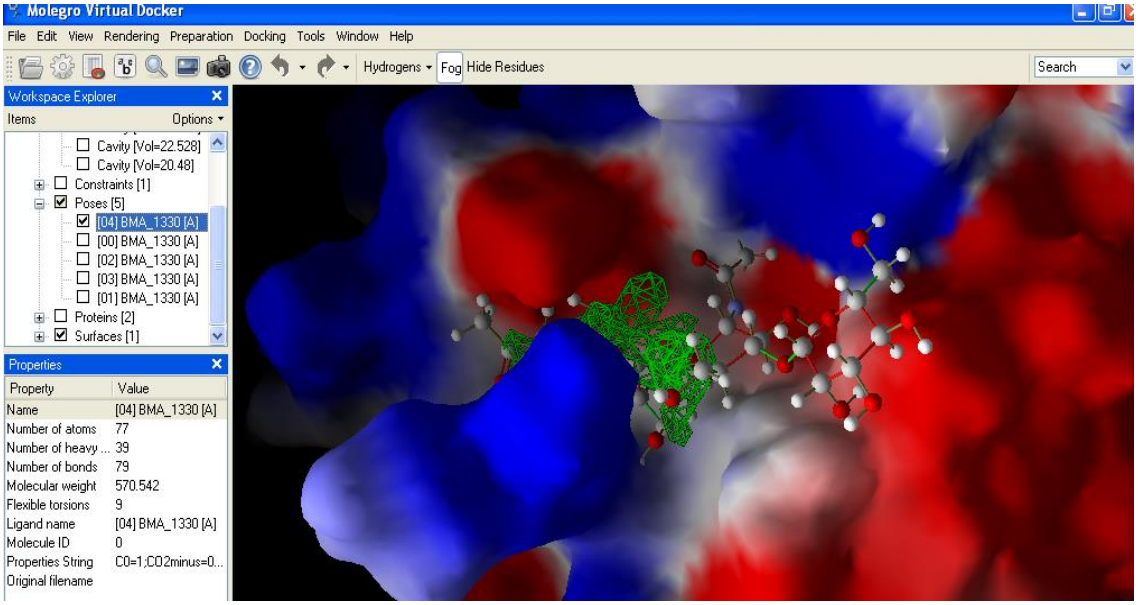


Inference: The 3D structure is shown in wireframe model with all the water molecules shown in red color.

Docking of TPI with EGFR using MOLGRO software:

Docking is one of the confirmatory step to conclude the inhibitory effect of TPI on EGFR receptor. The structure of EGFR was retreival from PDB. The 3D structures were used for docking using MOLGRO software and the results were analyzed.

Fig 9: Docking of TPI with EGFR receptor in MOLGRO



Inference: The above picture shows the docking image of EGFR shown in blue color to TPI shown in ball an stick model. The results of dockign indicate that the best docking pose for the above pair is the pose 00 with its score of -126.705. Thus proving the potential of TPI gene in inhibiting cancer cell receptor EGFR thus controlling cell proliferation.

Conclusion

Based on the above studies it can be concluded that Microbial TPI up regulates the proliferation of human cancer cells based on docking studies. This regulation is facilitated by its docking with EGFR as the cancer receptor with which it shows good docking score of -126.705. Isolation and identification of *Lactobacillus delbrukii* was done from milk and curd source following several biochemical tests and Gram's staining. A successful transformation of *Lactobacillus delbrukii* TPI into mutated *E coli* strain was also accomplished in this work by virtue of which the strains became able to grow in glucose media.

References:

1) Bartkiene E, Özogul F, Rocha JM. Bread Sourdough Lactic Acid Bacteria-Technological, Antimicrobial, Toxin-Degrading, Immune System-, and Faecal Microbiota-Modelling Biological Agents for the Preparation of Food, Nutraceuticals and Feed. Foods. 2022 Feb 3;11(3):452. doi: 10.3390/foods11030452. PMID: 35159602; PMCID: PMC8834576

2) Bintsis T. Lactic acid bacteria as starter cultures: An update in their metabolism and genetics. AIMS Microbiol. 2018 Dec 11;4(4):665-684. doi: 10.3934/microbiol.2018.4.665. PMID: 31294241; PMCID: PMC6613329.

3) Darbro BW, Petroelje BK, Doern GV. Lactobacillus delbrueckii as the cause of urinary tract infection. J Clin Microbiol. 2009 Jan;47(1):275-7. doi: 10.1128/JCM.01630-08. Epub 2008 Nov 5. PMID: 18987176; PMCID: PMC2620876.

4) El Kafsi H, Binesse J, Loux V, Buratti J, Boudebbouze S, Dervyn R, Kennedy S, Galleron N, Quinquis B, Batto JM, Moumen B, Maguin E, van de Guchte M. Lactobacillus delbrueckii ssp. lactis and ssp. bulgaricus: a chronicle of evolution in action. BMC Genomics. 2014 May 28;15(1):407. doi: 10.1186/1471-2164-15-407. PMID: 24884896; PMCID: PMC4082628.

5) Feyereisen M, Mahony J, Kelleher P, Roberts RJ, O'Sullivan T, Geertman JA, van Sinderen D. Comparative genome analysis of the Lactobacillus brevis species. BMC Genomics. 2019 May 23;20(1):416. doi: 10.1186/s12864-019-5783-1. PMID: 31122208; PMCID: PMC6533708.

6) Kashyap, A., Gunjan, V. K., Kumar, A., Shaik, F., & Rao, A. A. (2016). Computational and clinical approach in lung cancer detection and analysis. Procedia Computer Science, 89, 528-533.

7) María García-Burgos, Jorge Moreno-Fernández, María J.M. Alférez, Javier Díaz-Castro, Inmaculada López-Aliaga, New perspectives in fermented dairy products and their health relevance, Journal of Functional Foods, Volume 72,2020,104059,ISSN 1756-464, https://doi.org/10.1016/j.jff.2020.104059.

8) SuryaNarayana, G., Kolli, K., Ansari, M. D., & Gunjan, V. K. (2021). A traditional analysis for efficient data mining with integrated association mining into regression techniques. In ICCCE 2020 (pp. 1393-1404). Springer, Singapore.

9) Shimada A, Noda M, Matoba Y, Kumagai T, Kozai K, Sugiyama M. Oral lactic acid bacteria related to the occurrence and/or progression of dental caries in Japanese preschool children. Biosci Microbiota Food Health. 2015;34(2):29-36. doi: 10.12938/bmfh.2014-015. Epub 2015 Jan 30. PMID: 25918670; PMCID: PMC4405395.

10) Wierenga RK, Kapetaniou EG, Venkatesan R. Triosephosphate isomerase: a highly evolved biocatalyst. Cell Mol Life Sci. 2010 Dec;67(23):3961-82. doi: 10.1007/s00018-010-0473-9. Epub 2010 Aug 7. PMID: 20694739.

11) Wilson K. Preparation of genomic DNA from bacteria. Curr Protoc Mol Biol. 2001 Nov;Chapter 2:Unit 2.4. doi: 10.1002/0471142727.mb0204s56. PMID: 18265184.