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Isolation Characterization And Production of Extracellular Keratinase Enzyme from Bacillus Subtilis and Its Application in Industries

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Abstract:- The aim of this present study was to isolate the keratinase producing bacteria from poultry farm and slaughter house. In that, feather dumping soil (100 samples) was collected from several area includes the Coimbatore, Pollachi, and Erode. Among these samples, the eighteen isolate were subjected for primary screening using Milk casein agar plate. From these isolates, the Bacillus subtilis showing a highest keratinase activity in secondary screening using Casein agar medium & it was confirmed by 16s rRNA molecular sequence. The specific organism subjected to keratinase activity assay and identified by the spectrometric using the keratin azure as a substrate.The enzyme were undergone with the standard Purification process, its includes the ammonium sulphate precipitation, DEAE Sephadex K-50, Biogel G-200.The molecular mass of specific protein is 40KDa. There are enomorous types of azo dyes were classified and connected to this three types of azo dyes were used, which include Kerria Lacca, Rubia cardifolia, Accacia catechu. The newly isolated Bacillus subtilis can able to decolour the Rubia cardifolia effectively. According to the wet lab analyses , the OD value is 7.31 (Rubia cardifolia). In dry lab analysis, the "G" score value about -4.01; which indicates that there is an higher indication profile of keratinase with alizarin.

Keywords: *Soil sample, Bacillus subtilis, Azo dye, alizarin.*

I. INTRODUCTION

Feathers are the globally generated in bulk quantity as a byproduct from the poultry industry. It has been estimated that 400 million chicks are processed every week worldwide, the daily accumulation of feather waste reaches 5 million tons[1].Feathers make a serious problem as environment pollutant and in outbreak of H5N1 viruses[2].It has a very rich source of protein with beta keratin[3],and it contains about 90% of crude protein in the form of keratin[4]. Feathers represent 5-7% of the total weight of mature chicken [5]. These feathers were come around by several different approaches have been used for disposing the feather waste, including land filling, burning, natural gas production, nitrogen fertilizing for the plants[6] and treatment for animal feed but still it constitute sizable waste disposal problem[7]

Feathers hydrolyzed by mechanical or chemical treatment can be converted to feedstuffs, fertilizers, glues and foils or used for the production of amino acids and peptides[8].This enzyme increased digestibility of a commercial feather meal up to 82% and could replace up to 7% of the dietary protein for growing chickens[9].It was concluded that not only feather meal (keratin) could be used as protein for animal food but also the biomass of the enzyme producing strain as well. These keratinolytic enzymes may have important applications in biotechnological and industrial processes involving keratin-containing wastes from the poultry and leather industries through the development of nonpolluting processes and dehairing of skin and hides [10].Huge amount of this protein are available as feathers, a waste by the poultry production. Keratin occurs in nature mainly in the form of hair, feather, wool, horn and nail [11].Goat hair, sheep wool and buffalo horn showed lower response towards materials are abundant in nature, but have limited uses in practices since they are insoluble and resistant to degradation by the common proteolytic enzymes. Keratinase wastes represent a source of valuable protein and amino acid [13]. Diaminoacid cysteine makes an important contribution to the stability of protein, as it contains two α-carbonyl groups and can therefore form part of two adjacent polypeptide chains linking them together through the disulphide group of cysteine residue. In the case beta sheet, this allows sterially unhindered hydrogen bound between the amino acid carboxyl group of peptide bound, facilitating their close alignment and strong binding fibrous keratin. The molecules can twist around each other to form helical intermediate filament [14].

Basically, there are two types of keratin i.e.,α-keratin - in the form of folder chain and present in wool, hair and horn and β-keratin in the form of peptide chain in feathers. Based on the chemical structure the keratin is grouped into hard keratin (feather, hair, hoof and nail) and soft keratin (skin and callus) according to the sulphur content, which is as high as 5% in the case of hard keratin as low as 1% in soft keratin.Hard keratin like chicken feather which are recognized as a solid waste generated from poultry processing industry are insoluble and resistant to degradation by common proteolytic enzyme [15]. Keratin is insoluble in water, aqueous solution of neutral salt and organic solvents. However, much current research is centered on the potential use of keratinase of bacterial origin for the industrial compounds [16]. Keratinase material is important in medical, agricultural and biological [17], biodegradation films [18], prion protein [19],conversion of keratin waste into biohydrogen [20], woolen textile industry [21], detergent application [22].

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Till now, more than 30 keratinase producing bacterial culture were purified, characterized, and are widely used in various industrial purpose. The species of Bacillus sp., belongs to family Bacillaceae is widely distributed in all major geographic region of the world, but less explored studied. According to environmental pollution strategy, the ongoing research activities about discoloring the effluent from the dying industries. Basically, the dye consists of active or azo dye and reactive dye. There are enomorous types of azo dyes were classified and connected to this three types of azo dyes were used, which include Kerria Lacca, Rubia cardifolia, Accacia catechu. This work is a myriad of noval application have been studied, which is useful in industrial application to controlling the environmental pollution.

II. MATERIALS & METHODS

A. Isolation of keratinase producing microorganism

- Collection of soil sample

In this study, 300 numbers of soil sample (100 gm) were collected from natural composting waste and in poultry shops located at different places in Coimbatore, Erode, and Pollachi. Each 100 gm of soil sample was taken in a sterile plastic bag and it was transported to the laboratory.

- Isolation of Bacteria

Serial dilution from each sample was prepared by adding 1 gm of the soil sample to 100ml of distilled water (10-2 dilution). Samples were inoculated into standard procedure. On the other hand, the nutrient agar medium was prepared and the samples were inoculated under spread plate technique and incubated at 37°C for 24hrs. After incubation, the individual colonies were again inoculated with the nutrient agar medium.

- Screening for keratinolytic bacteria

To isolating the keratinase producing bacteria, the individual colonies are again inoculated with the casein agar medium. A total of 35 positive strains were isolated and ability to produce keratinase was studied in liquid media (Feather meal agar). Only one sample shows clearness about 25mm (casein agar medium) & its indicating maximum keratinase production. The positive colonies from this casein agar medium, it was checked with morphology, biochemical characteristic and in order to identify the keratinase producing bacteria and it was act as a preliminary identification.

- Identification and 16S rRNA sequencing and phylogenic Analysis

For species level of molecular identification was done by the 16S rRNA using standard primer, phylogenetic tree was constructed with MEGA online and it was submitted to NCBI-FASTA.

III. APPLICATION

A. Azo dye decolorized by the newly isolated keratinase enzyme

- *Azo dye (Wet Lab Analysis)*

Mother Nature has gifted us with more than 500 color giving plants. The azo dyes are available in abundance cost effective, yield good color and fastness properties. In this research work, three type of azo dye were used, it includes Nimbus, Rubia, Bee (Trade name).

- *Collection of effluent*

10ml of effluent was collected in a sterile plastic container in common dying industries at Tripur.

- *Azo dye decolorizing*

The effluent was taken into the laboratory and kept for 24 hrs to check, there is any change in colorations. After an over a period of 24 hrs, the newly isolated keratinase enzyme was inoculated into effluent and kept for 48 hrs. Thus, the color change in the effluent was observed in the UV Spectro photometer at 280nm.

- *Azo dye (Dry Lab Analysis)*

  - *Protein Sequence search using GenPept*

The National Centre for Biotechnological Informatics (NCBI) fosters GenPept (http//www.ncbi.nlm.nih.gov/protein) that coordinates with individual laboratories and other sequence databases such as PIR. GenPept is a genetic sequence which contains translated protein-coding sequence which is associated with each CDS through the addition of a translation qualifier in the Genbank feature table. The GenBank amino acid sequence is simply copied from the value searching with existing software and maintained because its data format is useful for similarity searching with existing software. GenPept is not an official release from NCBI but is thoroughly maintained and synchronized with each new release of Genbank. GenPept format is textbased multisequence format and can be derived from the parent GenBank format.

  - *Protein Structure prediction using SchrodingerSuite*

Prime 2.1 is a highly accurate protein structure prediction suite of programs that integrates Comparative Modeling and Fold Recognition into a single user-friendly, wizard-like interface. The Comparative Modeling path incorporates the complete protein structure prediction process from template identification, to alignment, to model building. Refinement can then be done from a separate panel, and involves side-chain prediction, loop prediction, and minimization.

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- *Docking of targeted protein and ligand*

Maestro is the graphical user interface for nearly all of the products that Schrodinger distributes: CombiGlide, ConfGen, Desmond, Epik, Glide, Impact, Jaguar, Liaison, LigPrep, Macro-Model, Phase, Prime, PrimeX, QikProp, QSite, SiteMap, Strike, and WaterMap. It contains tools for building, displaying, and manipulating chemical structures; for organizing, loading, and storing these structures and associated data; and for setting up, monitoring, and visualizing the results of calculations on these structures. For this study, Glide used to generate grid and interact the isolated ligand, Macro model used to simulate the complex protein.

- *Preparation of Protein and Ligand*

Retrieved structures were subjected to removal of Water upto 5 A distances, conversation of Selenomethionine into methionine, Selection of monomer to interact, assigning bond order in both polar and non polar amino acids, assigning lone pair electron atom, created disulfide bridges, filled side chains as the norms of crystally studied protein may have missing side chains, filled loop within the active regions in the protein and addition of hydrogen bond in the hydrophobic and hydrophilic amino acids. These parameters processed from Schrodinger Suite 2013-Protein Preparation Wizard and were made viable to interact with derived group. pH of 14 isolated compounds was between 7.0 to +/- 2.0 and retained chirality and original binding state of the compound.

- *Interaction of Targeted Protein and Isolated Liagnd*

Glide ligand docking jobs organized with 7 calculated protein grids and 14 ligand structures. Corrected Lewis structure was generated for ligand. Glide uses a hierarchical series of filters to search for possible locations of the ligand in the active-site region of the receptor. The shape and properties of the receptor are represented on a grid by several different sets of fields that provide progressively more accurate scoring of the ligand poses. Conformational flexibility is handled in Glide by an extensive conformational search, augmented by a heuristic screen that rapidly eliminates unsuitable conformations, such as conformations that have longrange internal hydrogen bonds. Each rotamer group is attached to the core bya rotatable bond, but does not contain additional rotatable bonds. The core is what remains when each terminus of the ligand is severed at the "last" rotatable bond. Carbon and nitrogen end groups terminated with hydrogen (—CH3, —NH2, —NH3 +) are not considered rotatable because their conformational variation is of little significance. Schrodinger's proprietary GlideScore multiligand scoring function is used to score the poses.

IV. RESULTS AND DISCUSSION

A. Isolation of keratinase producing microorganism

- Collection of Soil Sample

The 300 number of soil samples (100gm) were collected from the natural composting waste and in poultry shops located at different places in Coimbatore, Erode and Pollachi during January 2010. Soil samples were collected from 3 to 4 cm depth and transferred in sterile plastic bags. Collected samples were brought to the laboratory and use for the isolation of micro organisms.

- Isolation of Bacteria

Isolation of Bacteria was performed by serial dilution and plating method on nutrient agar medium (NAM). Serial dilution was preferred based on the standard technique and followed by spread plate method also was carried out.

(A)10-2, 10-3, 10-4

Dilution plate

(B)10-2, 10-3, 10-4

Dilution plate

The bacterial isolates were further subcultured on NAM to obtain pure culture.Pure isolates were maintained in NAM slants at 4°C for further studies.

Fig., 4.3 Streak Plate Method

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- Screening for Keratinolytic bacteria

From the pure culture, the isolated bacteria were again inoculated into casein agar medium (CAM) as the primary screening method. The pH was adjusted to 8.0.

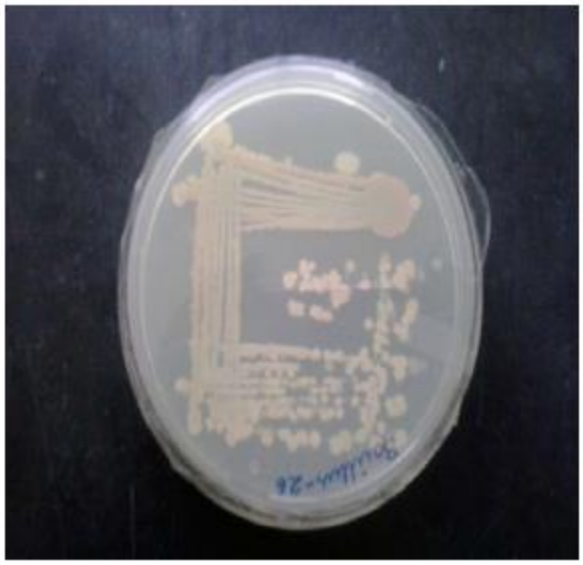

Fig 4.4. Specific Media- Casein Agar Medium (Primary Screening)

Amoung the 35 positive strains which was isolated from casein agar medium, only one strain shows a clear zone of hydrolysis about 25mm in dm and indicating that compared to other strains, thus it can able to secrete a higher amount of keratinase enzyme

Fig 4.5. Zone of Hydrolysis – Casein Agar Medium

Thus isolated strains were undergone with the secondary screening method. To obtains the optimum condition for maximum enzyme production in feather meal broth medium containing sterile feather as a substrate.

Fig 4.6. Feather Meal Broth (Secondary Screening)

B. Identification and 16S rRNA Sequencing and Phylogenetic Analysis

- *Identification of Bacteria*

Identification of the feather degrading isolate was carried out by studying the microscopic, cultural and biochemical characteristic according to the Bergey's manual.

C. The specific organism is confirmed by its Biochemical characteristic is as follows

S.No Bio-Chemical Test Result

1. Gram staining -Gram positive
2. Shape -Rod shape, raised, dull, opaque, grayish white, frosted glass appearance
3. Capsule -Present
4. Size -23 dm
5. Indole production -Negative
6. Methyl red -Negative
7. Vogesprousker -Positive
8. Citrate -Positive
9. Oxidase-Negative
10. catalase -Positive
11. Litmus milk reaction- Alkaline
12. Hydrogen sulphide -Negative
13. Urease test -Negative
14. Starch hydrolysis -Positive
15. Casein hydrolysis- Positive
16. Fructose & Glucose fermentation

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Test-Acid production ;no gas Based on these result, the postivie strain was identified as Bacillus sp.,Similar result was reported by (Williams et al., 1990), found a straight rod shape aerobic and endospore forming bacterium appeared in single or in chain catalase positive and gram variable classified as Bacillus sp. This bacterium isolated from high temperature poultry waste digest and able to degrade feather keratin when using feather as a primary source of carbon and energy. Atalo and Gashae 1993 described thermophilic Bacillus sp, produce protease to degrade various s fiber proteinsfeather hair sheep skin horn.

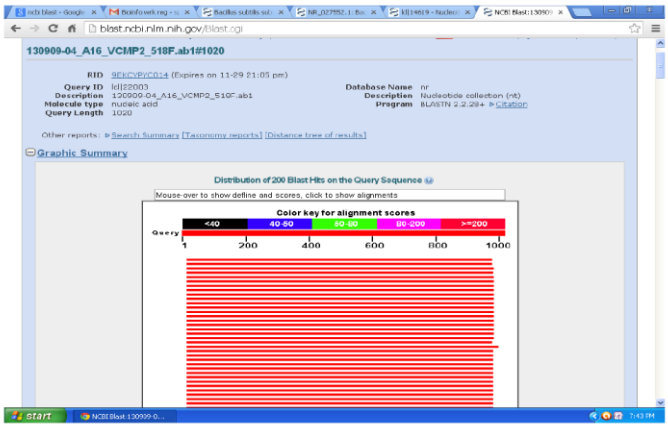
- *16S rRNA sequence*

For further identification by 16S rRNA sequencing, the genomic DNA (gDNA) was extracted and according to the standard bacterial gDNA extraction protocol. (Ausubelet al.,1995). Thus, the 16S rRNA sequencing analysis (revealed that the feather degrading isolate was found to have maximum similarity (95%) with the strain Bacillus subtilis.

Fig., 4.1 16s rRNA GENE SEQUENCE

- *Phylogenic Analysis*

The Phylogenetic tree was constructed using the MEGA online tool revealed the genetic relationship between the other species having Keratinase activity. The sample sequence was submitted to NCBI- FASTA with Accession Number:- N-10273.1



V. APPLICATION

A. Azo dye decolourizing by the newly isolated keratinase enzyme:-

- Azo dye (Wet Lab Analysis)

After over an period of hours, the color change in the different was noted. Based on the dye characteristic, the Rubia dye was decolouratized efficiently while it compared to other two dye, because Rubia dye is an natural oldest dye. The decolouration is also resulted in molecular modelling, the result is as follows. From these results, the keratinase enzyme was isolated from the Bacillus subtilis under chicken feather meal broth as a substrate under optimum pH 8 with temperature 50°C. The molecular weight of the enzyme was 30 KDa. Finally, the rubia dye effluent can be easily decolouring by this keratinase enzyme under sufficient period with optimum pH and temperature.

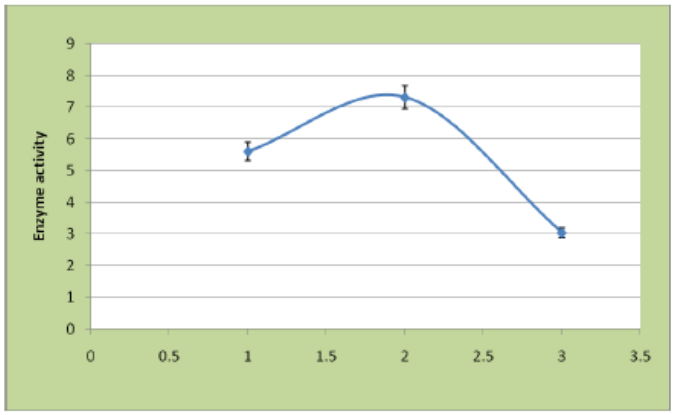


Fig. 4.30 Azo Dye decolourizing by keratinase enzyme

- *Azo dye (Dry Lab Analysis)*
  - Sequence Retrieval

Keratinase protein (367 residues) was retrieved from NCBI GenPept with the accession ID of AEI83225.1.

- TARGET PROTEIN

>Keratinase**[**AEI83225.1**]**
SSTDYVPNQLIVKFKQNASLSNVQSFHKSVGAN
VLSKDDKLGFEVVQFSKGTVKE
KINSYKNNPDVEYAEPNYYVHAFWTPNDPYFK
NQYGLQKIQAPQAWDSQRSDPG
VKVAIIDTGVQGSHPDLASKVIYGHDYVDNDN
TSDDGNGHGTHCAGITGALTNN
SIGIAGVAPQTSIYAVRVLDNQGSGTLDAVAQG
IREAADSGAKVISLSLGAPNGGT
ALQQAVQYAWNKGSVIVAAAGNAGNTKANY
PAYYSEVIAVASTDQSDKKSSFST
YGSWVDVAAPGSNIYSTYKGSTYQSLSGTSMA
TPHVAGVAALLANQGYSNTQIR
QIIESTTDKISGTGTYWKNGRVNAYKAVQYAK
QLQENKAS

- *Homology Modeling of Protein Sequence Retrieved*

Retrieved sequence was the subjected to perform Homology modelling against Protein Databank which has only structural conformation of each atom's configuration. Homology Modelling followed to progress based on Crystal studied structure as template structure. Structure Prediction was carried out using Schrodinger Prime Module for Target protein with the corresponding template id of protein 3WHI. The modeled structure was showing Residues in most favoured regions more than 90% for each modelled protein.

- *Docking Result*

Out of above mentioned G Score (Table 1) list, interaction profile of keratinase with alizarin showed interaction profile that the ligand interacted at 2 residual sites of the Protein having residual atom types ASP (H) receptor via O(Oxygen) atom types forming hydrogen bonds with the bond distance of 1.73A, 1.61 A, respectively. The higher interaction of keratinase with alizarin can be noticed from the Glide score; -4.01 as higher Interaction profile .

Tab., 4.19 "G" SCORE TABLE
1 alizarin -4.01
2 Diazol cutch F -3.95
3 lar1 -2.05

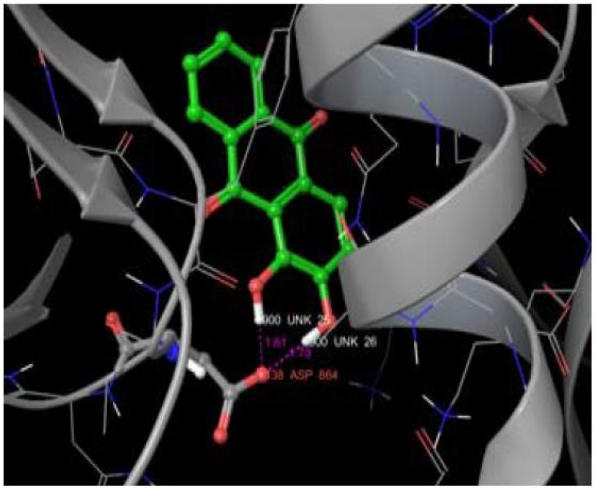


Fig. 4.32 Interaction profile for alizarin an keratinase obtained after molecular docking

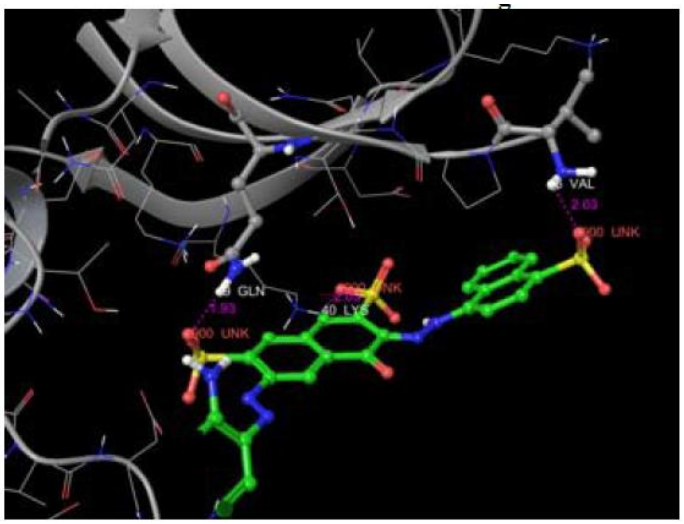


Fig. 4.33 Interaction profile for Diazol cutch F and keratinase.

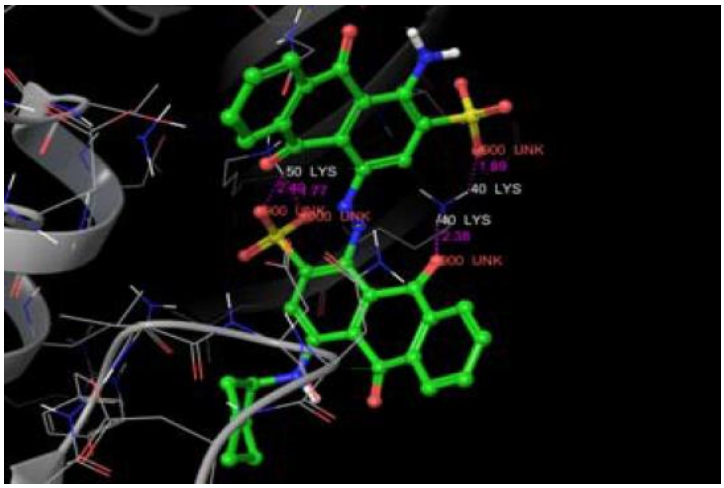


Fig. 4.34 Interaction of lar1 and keratinase.

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VI. CONCLUSION

Based on the free energy calculation of 'g' score justifies alizarin has higher binding affinity to keratinase protein 367 residuesdocking score. Thus, the higher interaction of keratinase with alizarin were -4.01 (Glide score) noted, while compared with the Diazol cutch F(-3.95) and Lar 1 (-2.05). Generally, the Glide score is as follows., ">>>" given a configuration of protein and the ligand and "<<<" returns a number representing "goodness" or "energy" of the configuration by Hongjian Li mail et al., (2014). For Glide SP or HTVS, scores of -10 or lower usually represent good binding. For some targets (e.g., with shallow active sites or predominantly hydrophobic interactions), scores of -8 or -9 might be very good. Metalloproteins and Glide XP both tend to produce lower GlideScores (-12 or below). The cutoff for a "good" score will vary a little from system to system, and changing the van der Waals radii scalings can change the scores significantly (Hawkins et al., 2010). Curcumin derivatives which are very potent antioxidant, free radical scavenger and known inhibitor of dioxygenases have been extensively studied to explore their potential utilization in chemoprevention. Docking analysis of curcumin derivatives: Tetrahydrocurcumin (THC), Bisdemethoxy curcumin (BDC). By using AutoDock 3.0, BDC showed better affinity with all anticancer therapy targets than THC. Interaction of BDC with respect to Matrix Mettaloprotease (MMPs) is represented. A docking energy of -11.46 Kcal/mol with three hydrogen bonds was showed. The hydrogen bond was formed between hydroxyl (H14) of the phenyl ring and carboxyl (O) of hydrophobic amino acid Pro421 by a distance of 2.114 A (O-H. . . O) and energy of -0.374 Kcal/mol. Another interaction bridging (O5) of the heptane branch and amine (NH2) of positively charged residue Arg424 with a distance of 1.793 A (N-H. . .O) along a minimum energy of -5.492 Kcal/mol. The hydroxyl (H74) of another phenyl ring and carboxyl (O) of hydrophobic amino acid Pro 430 by a distance of 1.858 A(O-H. . .O) along with a energy of- 1.817Kcal/mol Girija et al., (2010).

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