INTRODUCTION

Skin is a complex organ and known as the largest tissue of the body. It is fortuitous to be exposed with several vital and extraneous agents. The "epidermal melanin unit" facilitates the fortification of entire system by unblemished mechanism that is proceeded by the keratinocytes and melanocytes (Kunitakik, 2002; D’Mello et al., 2016). Melanin contributes for pigmentation in the physiology of an organism and is influenced by several internal and biological parameters. Physical agent like UV light affects externally whereas inherited traits and hormones create an internal impact. Melanogenesis is a multi-phased natural process which utilizes tyrosinase as the foremost enzyme and administers microphthalma-associated transcription factor thereby induces tyrosinase-related protein (TRP1 and TRP2) as major targets of melanogenic enzymes. (Kim et al., 2013). Melanin is an active pigment that possesses multiple properties such as photoprotective, photosensitive and antioxidantic. It participates in various physiological activities and primarily involves in protecting skin. The irregular functioning of melanin production indulges in indiscretion leading to hyperpigmentation of the skin. Though the pathological consequence is not much offensive yet it can cause significant cosmetic deformities which becomes a psychosocial burden for the patient. Apparently, this leads to diminutions in social functioning and low self-esteem (Chren et al., 2001).

Contemporary research associates melanogenesis disorders with progressive degeneration of nervous system that leads to several disparity in cognitive and motor neuron. Hence, the need for developing inhibitors to target multiple ailments has to be invigorated (Hara et al., 2000). Cosmetic industries target the regulation of melanogenesis as the principal requisite to improve fairness and skin texture. Fabrication of cosmetic products requires a precise mechanism that targets the production of melanin without offering side effects to the cells. Tyrosinase are considered as target to lessen the melanin production and thus increasing fairness in skin. Hence innovative skin colour lightening agents are on demand and several researches are initiated to generate them on large scale using various natural resources. Current scenario investigates varied phytochemical constituents and microbial proteins as sources. There are very few sightings that endorses tyrosinase as cosmetic targets to inhibit melanogenesis. Due to the undefined molecular mechanism and interaction of proteins in intracellular signalling pathways there is a necessitate to establish tyrosinase as direct inhibitors of melanogenesis in the cosmetics industry (Lin et al., 2012). Soil derived bacterium like Bacillus subtilis establish themselves for producing extracellular protein tyrosinase. It is a protease family derivative which facilitates a protective attribute in the microorganism by defending other evading pathogenic microorganisms. (Siezen et al., 1997; Nakjiang et al., 2012; Duarte et al., 2016). Thereby enhance vital physiological functions such as cell growth, post translational processing of hormones (Rockwell et al., 2004), and defending the organism with the presence of digestive protease like subtilisin. (Withers-Martinez et al., 2004). They are mainly involved in hydrolysis of the peptide bonds (boer, 1991; Lowe, et al., 2015) enduring high commercial importance that initiates nucleophilic attack on peptide bond through a serine residue located at active site. Subtilisin is a water-soluble proteolytic enzyme, whose application is widely used as a cleansing agent as cleansers at large scale industries and households. Alternatively, very few research supports the participation of subtilisin as protein hydrolyse production in both leather and cosmetics industry (Gupta et al., 2002). The pharmaceutical attribute of subtilisin holds a promise for its stability and therapeutic potency. It has the ability to digest and degrade gluten and can prevail amongst high acidic environment of stomach (Darwish et al., 2019). The reports on toxicity studies exhibited a LD50 value was 1.8 g/kg. The substantiated clinical studies of subtilisin incited on animals revealed gastrointestinal disturbances which may be due to its proteolytic feature (Figueiredo et al., 2018). Hence, pharmacologically engineered subtilisin with reduced protein degradation can develop ideal skin care potential. The application of this substantial protein in skin-care diligence is at miniscule and hence there is a need of much research to hoist its potential as a cosmetic agent. This study has implemented subtilisin isolated from Bacillus subtilis as a lead candidate with the potential of regulating melanogenesis.

MATERIAL AND METHODS

Soil sample collection and preparation

The soil sample was collected from Anikurunthan, Ramanathapuram district, Tamilnadu, India. Approximately 5 g of soil was collected and transferred to lab
under sterile conditions. Further, 1 gm of constituent was supplemented to 5mL of nutrient broth. This suspension was incubated at 35°C for 24 hours

Isolation and characterization of Bacillus sp.

Subsequently, the revived sample was serially diluted and plated on nutrient agar plates constituted with 20% raw milk. After a day’s incubation the morphological (staining) and chemical attributes (biochemical test) of the isolate was identified based on Bergey’s Manual of Systematic Bacteriology (Bergey and Holt, 2000). A series of identification tests such as motility, starch hydrolysis, Gelatin hydrolysis, Casein hydrolysis, Citrate utilization, Voges-Proskauer VP, Methyl red (MR ), catalase, nitrate reduction, Triple sugar agar, Carbohydrate fermentation were performed.

Production and assay of Subtilisin

The soil isolate was fortified to produce subtilisin by exposing the culture to a standard media constituting the following composition: yeast extract 6.75g/l, peptone 4.41g/l, sodium chloride 6.08g/l, casein 10.75g/l and glucose 5.00g/l. At an optimum pH 7.5 in room temperature the culture was standardised subtilisin production. Though, subtilisin isolated from Bacillus sp. is considered to be extracellular, the intracellular constituents is also validated in this study. The conventional Anson method modified by (Yang and Huang, 1994; Mokashe and Patil 2016) assay using casein as substrate was followed in the study. The quantification of the product was validated using the following equation by calculating the tyrosine concentration using the regression equation obtained from its standard curve (Navaneeth et al., 2009; Boeckx et al., 2017).

\[ \text{Tyrosine concentration (µg/ml)} = \frac{\text{Test O.D} - \text{Control O.D}}{0.285} \times R^2 \]

The overall protein component present in the product was estimated using the standard Lowry’s method (Lowry et al., 1951). The specific constituents of subtilisin was detected and validated with a HPLC run (Perkin Elmer Corp. Norwalk, U.S.A) equipped with UV detector at standard absorption of 220 nm. The isoelectric point was applied to C-18 column with an injection volume of 10 ml using 59% of Acetonitrile and 0.1% Trifluoroacetic acid as an eluent solvent.

Tyrosinase Assay

B16F10(15C® CRL-6675™) cells were purchased from Hi media laboratories (Mumbai, India) and used for in vitro studies with standard cultural conditions. The analysis was performed by the protocol reported by (Baurin et al., 2002; Dong et al., 2020) with modifications by adapting the typical UV absorption studies at 475 nm.

In-silico Analysis – Molecular Docking

Molecular docking was performed using GRAMMX web server. The target proteins and ligands were prepared before initiating docking.

Peptide sequence retrieval and toxicity prediction

The sequence of subtilisin from Bacillus sp with Id AOR97460.1, was retrieved from the NCBI protein database and checked for its toxicity using toxin-pred tool by breaking it into peptides.

Target Protein sequence retrieval

The sequences of target proteins namely TRP1 and TRP2 alternatively called Microphthalmia associated transcription factor were retrieved from Uniprot with the Ids Q60722 and O75030 respectively.

Target protein structure prediction

The 3D structure of TRP1 was downloaded from PDB since it was available in the ID 5MBP. There were no 3D structures available for TRP2 and Subtilisin and hence they were comparatively modeled using Swiss Model and Raptor X tools respectively.

Structure validation

The modelled structures of subtilisin and TRP2 were validated with Ramachandran plot using SAVS server.

Protein - Protein Docking

The target protein structures TRP1 and TRP2 were docked against the subtilisin using GRAMMX web server to check its interaction and binding. To browse the binding sites, protein and ligand (protein) was fixed in the docking software. The docking parameters were set as (200)/ (70) for Population size and Number of generations. Docking process started by analysing the interactions of the target and ligand. The results were viewed through docked poses to get interaction profile and interaction analysis in the table format.

RESULTS AND DISCUSSION

Isolation of bacteria

The bacteria were isolated from soil sample in a nutrient agar medium with 20% of raw milk and zone formation was observed around the colonies (Fig 1), thus indicates that the bacteria have the capacity of producing proteolytic enzymes and extracellular compounds. The identification of the isolates was preliminarily determined in accordance to Bergey’s manual (Berkeley et al., 1984). Bacillus species are diverse with the presence of endospores which is considered as a foremost adaptation against heat. These species are selectively grown on media nourished with raw milk. Figure 1 illustrates the structural features of the isolate where the identifications were based on its colour. The Morphological characterization was based on classical macroscopic techniques of colour, form, shape, and elevation of pure colonies and distinct growth patterns. Table 2 provides a clear morphological description of the isolate.

![Figure 1](image)

**Table 1 Morphological features of isolated bacteria**

| Colony Morphology | Circular | Convex | Even | Smooth | Rough | Half-White (Cream tint) | Dense | + | Rod |
|-------------------|---------|--------|------|--------|-------|------------------------|-------|---|-----|
| Framework         |         |        |      |        |       |                        |       |   |     |
| Border            |         |        |      |        |       |                        |       |   |     |
| Elevation         |         |        |      |        |       |                        |       |   |     |
| Exterior          |         |        |      |        |       |                        |       |   |     |
| Consistency       |         |        |      |        |       |                        |       |   |     |
| Color             |         |        |      |        |       |                        |       |   |     |
| Opaqueness        |         |        |      |        |       |                        |       |   |     |

Biochemical tests

The primary biochemical tests were done to ensure that the bacteria are Bacillus sp. The table 2 shows the positive and negative results based on the biochemical activity of the isolated bacteria. The biochemical test results along with results from ABIS online software confirmed that the isolated bacterium was Bacillus sp. The series of tests depicted in table 2 augmented the capability of the organism to secrete both extracellular and intracellular enzyme. The structural organizational of the isolate was preliminarily recognized and were in concurrence to (Vargas et al., 2004). The present study was carried out to evaluate the production of subtilisin from a soil borne Bacillus species isolate.
Table 2 Biochemical tests for isolated bacteria

| Tests          | Result |
|----------------|--------|
| Catalase       | +      |
| Citrate        | +      |
| Gelatin hydrolysis | +   |
| Gram staining  | +      |
| Indole         | -      |
| Methyl red     | +      |
| Nitrate reduction | +  |
| Voges Proskauer| -      |
| Triple sugar agar | +    |
| Casein hydrolysis | +  |
| Starch         | +      |
| Glucose        | +      |
| Sucrose        | +      |
| Lactose        | -      |

Legend: ‘+’: Presence; ‘-’: Absence

Assay of subtilisin

Existing as a protein digesting enzyme subtilisin has the capacity to undergo hydrolysis by utilizing a substrate and breaking peptide bonds. Casom being the substrate is used by the protease activity there by increasing the free amino acid as end products. The polypeptide concentration is validated to assay the activity of the produced subtilisin. (Yang and Huang, 1994; Mokashe and Patil 2016). The amount of protein is related to the cleaved amino acid. In accordance to the habitat and physiology of microorganisms the quantity of protein varies. The quantitative assay performed exhibited higher enzymatic activity (520.82 U/ml) on casein induced nutrient agar medium (Fig 2) which is in accordance to (Yanga et al., 2000; Karadag, 2009).

Figure 2 Standard graph of tyrosine with the concentration of mg/ml

Protein estimation by Lowry’s method

The extraction process is followed by an estimation analysis and the extracted protein was estimated by Lowry’s method (Fig 3). The concentration of crude protein was calculated from the standard graph of BSA. The optical density of the samples at various concentrations revealed the amount of protein in the extract. The crude protein was quantified with 1mg/ml of standard whose value augmented for which the infinity was calculated with the regression coefficient. Subsequently in accordance to coefficient factor the quantity of protein was found to be 1.67 mg/ml. Fig. 3. There was a direct proportion between the intensity of the absorbance increase (y) and the amount of the protein (x) over the range 0-2.5 mg in a total volume.

Figure 3 Standard graph of protein and the concentration of BSA was mg/ml

HPLC Analysis

High-performance liquid chromatography is favoured to identify and separate bioactive compounds and biomolecules (Ottesen and Svendsen, 1970; Danafar and Hamidi, 2015). The analytical instrument is profoundly used in separating various organic, phytochemicals and microbiological proteins (Iklai, 1976; Strongin et al., 1978; Batrawi et al., 2017). This study evaluated the presence of subtilisin which is an alkaline serine protease. Intracellular (S1) and extracellular (S2) exudates of the bacterial isolates were taken as test samples. The HPLC chromatogram of intracellular exudate relatively has low number of peaks when compared to the extracellular sample. Active subtilisin hydrolyses the propeptide and can form strong subtilisin propeptide complex which could be demonstrated by HPLC analysis. According to the study of (Hu et al., 1996; Nakai et al., 2012) subtilisin was eluted at the retention time of 15.39 min which also formed a propeptide complex. In accordance with the above stated reference both the S1 (Fig 10) and S2 (Fig 11) exudates showed the peak with a retention time of 15.62 min and 15.0 min. When analysing the peak S1 exhibited a greater area percentage (5.36 %) with an increased height of 2289µV. Subtilisin E is an extracellular protein derived from Bacillus subtilis (Bryan, 1987; Bryan, 2000; Pannuk et al., 2015) but the chromatogram of S1 also showed similar retention time as that of S2. This may be due to certain alterations in the bacterial physiology due to stress. Though various studies have been performed on bacterial isolates with HPLC, specific profiles on subtilisin are very few. This may be due to the need for vast injection volumes for resolving low- frequent peaks with the analytical instruments. For a bacterial isolate specific protein recovery is a real challenge because of its low optical density and Gram-positive species face this difficulty intensively. Furthermore, there is a need to profile on several microbial model organisms. This study requires a standardization with a comparative profile using subtilisin as a reference sample. There is a profound necessary of a systematic comparison among different strains of organisms in order explore their novel metabolites.

Figure 4 Chromatogram of Intracellular Crude Content showing Subtilisin propeptide (a) complex from Bacillus sp.

Figure 5 Chromatogram of Extracellular Crude Content showing Subtilisin propeptide (b) complex from Bacillus sp.

Anti melanogenesis activity - Tyrosinase Inhibition Assay

Subtilisin has received significant attention as detergent and is known as stain cutter (Kirk et al., 2002). However, little research has focused on its antimelanogenesis activity and this in vitro assay relates its potential in cosmetic field. Tyrosinase is a vital enzyme that promotes melanin biosynthesis. However, the accumulation of melanin causes serious skin ailments where, there is a need of
anti-tyrosinase mechanism to exert skin protection and inhibit melanogenesis (Perluigi et al., 2002). B16F10 cells constitutes of intracellular tyrosinase, and can monitor the antimelanogenesis through in vitro assays. The inhibition of the tyrosinase in oxidation of L-DOPA as its substrate was assessed. The results indicate that Kojic Acid (1 mg/mL) effect on tyrosinase activity. Although the inhibition percent is relatively low than positive control it exhibited a regulating potential when compared to negative control and can be incorporated as an innocuous compound devoid of adverse effects.

TYROSINASE ASSAY

Figure 6 Tyrosinase Activity Assay in B16F10 cell lines in the presence of Kojic

| Peptide sequence* | Mutation position | SVM score | Hydrophobicity | Hydrophobicity | Charge | Molecular weight |
|-------------------|-------------------|-----------|---------------|---------------|--------|-----------------|
| MRSKKLWSSLFALTTMAFSNMSAQAA | No | -1.38 | 0.05 | 88 | -0.67 | 3 | 3392.59 |
| GKSSTEEKYYIVGFQTMSSAMKDKDIVS | No Mutation | -0.37 | -0.24 | -0.62 | 0.44 | 5 | 3266.27 |
| EKGGKVQKFQKYVNNAATLDEKAVKELK | No | -1.15 | -0.29 | -0.88 | 0.69 | 4 | 3321.33 |
| DPSVAWYEEHHAYFAQSPYVGIGQIKAP | No | -0.83 | -0.08 | -0.43 | -0.02 | -3 | 3315.05 |
| ALHSQGYTGNSKVAVIYSGIDHSPDNLNV | No Mutation | -0.84 | -0.06 | -0.1 | -0.13 | -1 | 3081.8 |
| RGGASFVPETNPYGSSHGTAVGTTAIA | No | -1.01 | -0.09 | -0.46 | -0.11 | 0 | 2972.57 |
| LNSSIVGVLAPASLYAVKVLSTGSGQY | No | -1.63 | 0.04 | 0.45 | -0.45 | 0 | 2981.81 |
| SWINGEWAISNNMDVINMLGPGTGSTA | No | -1.09 | 0.06 | 0.24 | -0.52 | -2 | 3150.01 |
| LKTVDVAKAVSIVVAAANNGEGSSGTST | No | -0.99 | -0.01 | 0.38 | -0.03 | 0 | 2764.47 |
| VGPAYKYPSTIAVGAVNNSQARFSASGS | No | -0.6 | -0.07 | -0.11 | -0.25 | 2 | 2974.65 |
| ELDMVPAPGSQSTLPGTYAGNTSMAT | No | -1.32 | 0.02 | 0.09 | -0.38 | -2 | 2989.77 |
| PHVAGAAAALILSKHPTWNAQVRDLLESTA | No | -1.04 | -0.13 | -0.21 | -0.09 | 2 | 3212.07 |
| TYLGSFFYKGLINVQAAA | No | -0.9 | 0.02 | 0.13 | -0.04 | 1 | 2251.84 |
| MRSKKLWSSLFALTTMAFSNMSAQAA | No | -1.38 | 0.05 | 88 | -0.67 | 3 | 3392.59 |
| GKSSTEEKYYIVGFQTMSSAMKDKDIVS | No Mutation | -0.37 | -0.24 | -0.62 | 0.44 | 5 | 3266.27 |
| EKGGKVQKFQKYVNNAATLDEKAVKELK | No | -1.15 | -0.29 | -0.88 | 0.69 | 4 | 3321.33 |
| DPSVAWYEEHHAYFAQSPYVGIGQIKAP | No | -0.83 | -0.08 | -0.43 | -0.02 | -3 | 3315.05 |
| ALHSQGYTGNSKVAVIYSGIDHSPDNLNV | No Mutation | -0.84 | -0.06 | -0.1 | -0.13 | -1 | 3081.8 |
| RGGASFVPETNPYGSSHGTAVGTTAIA | No | -1.01 | -0.09 | -0.46 | -0.11 | 0 | 2972.57 |
| LNSSIVGVLAPASLYAVKVLSTGSGQY | No | -1.63 | 0.04 | 0.45 | -0.45 | 0 | 2981.81 |
| SWINGEWAISNNMDVINMLGPGTGSTA | No | -1.09 | 0.06 | 0.24 | -0.52 | -2 | 3150.01 |
| LKTVDVAKAVSIVVAAANNGEGSSGTST | No | -0.99 | -0.01 | 0.38 | -0.03 | 0 | 2764.47 |
| VGPAYKYPSTIAVGAVNNSQARFSASGS | No | -0.6 | -0.07 | -0.11 | -0.25 | 2 | 2974.65 |
| ELDMVPAPGSQSTLPGTYAGNTSMAT | No | -1.32 | 0.02 | 0.09 | -0.38 | -2 | 2989.77 |
| PHVAGAAAALILSKHPTWNAQVRDLLESTA | No | -1.04 | -0.13 | -0.21 | -0.09 | 2 | 3212.07 |
| TYLGSFFYKGLINVQAAA | No | -0.9 | 0.02 | 0.13 | -0.04 | 1 | 2251.84 |

*Prediction - Non Toxic

Table 3 Toxicity Prediction of Subtilisin

In silico Toxicity Prediction of Subtilisin

There is an excessive commercial application of subtilisin and hence there is a significant need to assay its hostile effects. Evaluating the toxicity of a distinct or assembled lead molecules with animal models is a tedious long-term process that involves high expenditure (Valerio, 2009). In silico tools can significantly support toxicity assessment and can limit the number of clinical trials. It comprises potential approaches to assay the rate of toxicity in a compound by scheming related computational algorithms and programmes which can relatively reduce the need of animal studies (Ruas and Bajic 2016). The protein sequence of subtilisin that was retrieved from Uniprot was subjected to toxicity prediction using Toxinpred web server and the results are provided in table 3. The amino acid and peptide composition is assessed based on SVM model to discriminate toxic from non-toxic peptides. Where the resulted peptides were found to be non-toxic and there were no mutations found on the sequences. The showed good scores that ranged from -0.37 to -1.38 which is consistent with global binary models (Li et al., 2017). The molecular weights were was within 500KD which is an ideal mass for a drug.
Protein structure can be characterized by the typical values of the main chain torsion angles $\phi$ and $\psi$ which can be visualized by a bidimensional diagram called Ramachandran plot. Sequence identity was found to be 54.85% with the structure of modelled TRP2. Ramachandran plot predicted the structure (TRP2) to be more feasible with the percentage of amino acids in the favourable region was found to be 87% (Figure 9). The result of Ramachandran plot analysis supported the high-quality structure of the refined model.

**Protein - Protein Docking**

The molecular inhibitory interaction of subtilisin in the form of 3D structure (Fig 7&8) against melanogenesis inducing factors TRP1 and TRP2 was assayed in silico using molecular docking. Here, the protein subtilisin was considered as ligand and its interaction was found to be -675569 KJ/Mol,36957KJ/Mol and after minimization TRP1 and TRP2 were docked with subtilisin through GRAMMIX webserver. The ligands were found to be interacting well in the cavities with finest docked pose (Fig 10 &11). Molecular structure of a protein in 3-dimensional form is a significant feature in docking. Unfortunately, only few crystal structures of enzymes are reported (Kim et al., 2004). Hence there was a need to model the second targeted protein of our study (TRP2). Adapting computational structural dynamics tools the unknown structure of a protein can be elucidated. (Xu, 2013) which can be further endorsed by Ramachandran plot.

Protein interactions generally has the potential to form complexes with biomolecules (e.g. Ag-Ab, Enzyme-substrate complexes). The tertiary structure of proteins is necessary to understand the binding mode and affinity between interacting molecules. Protein interaction studies are deciphered through the tertiary features of proteins. Further using advance technology and computational simulation the 3-D structural (Santoyo et al., 2013). The software validating this docking event need to be more specific since the minimized energy confirmation is generally too large. This is due to the extensive size of proteins and when two of them interact massive amount energy is expelled. This study reveals one such assay where a high minimized energy is produced from both the targeted proteins. Hence this assay paves a baseline to elevate subtilisin as a lead compound in the field of drug discovery regulating melanogenesis. This can be substantiated by clinical trials which would elevate the standards of cosmetic industry.
CONCLUSION

Melanogenesis is considered as a vital physiological event due to its potential of absorbing light and performing a photoprotective role. Though there are merits in its physiological process accretion of melanin could lead to skin aberrations and deformity. Regulation of this process is considered as a significant target in skin industry., Hence this study considers tyrosinase-related protein (TRP1 and TRP2) as target proteins. Subtilisin an alkaline serine protease is prospected as a ligand. The prior ligand assay predicted subtilisin as a nontoxic interaction) was executed wi

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