



Enzymatic production of 4-cholesten-3-one using cholesterol oxidase from *Bacillus cereus* strain KAVK5 and its potentiality toward the inhibition of disease-associated proteins an *in silico* approach

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ABSTRACT

The biological conversion of cholesterol into 4-cholesten-3-one (4-CHN), with the help of commercially important cholesterol oxidase (CHO) originated in *Bacillus cereus* strain KAVK5 from ghee, has been explored in this study. 4-CHN is extensively studied in the field of medicine. This research work aimed to optimize CHO productivity from KAVK5, which is employed to produce 4-CHN. Qualitative and quantitative methods examined CHO production and the modification of the fermentation medium increased its productivity. It was optimized using components such as xylose, peptone, sodium nitrate, and manganese sulfate with pH 7.0 maintained at 30°C for 32 hours of incubation. KAVK5 markedly yielded a superior level of CHO (1.86 U/ml) in an optimized media, and the molecular weight was achieved to be 32.8 kDa. In addition, the bioconversion process was implemented by the double-phasic system, i.e., aqueous and organic. The report affirmed that 1.23 g of 4-CHN was yielded for every bioconversional process. An *in silico* docking study was carried out using the receptors associated with obesity, diabetes, cancer, and bactericidal disorders. The outcome of this report declared that an activated ligand 4-CHN is treated as the best therapeutic agent to treat diseases.

INTRODUCTION

Cholesterol is an integral substance found in the human body. Globally, inflated serum cholesterol has developed into a significant lifestyle-oriented disorder that is raised with high morbidity and mortality (Singhal *et al.*, 2019). Major concentration has been focused on drug therapy. Nonpharmacological activity includes dietary intervention, behavior modification, and regular exercise, which are commonly pursued to lower blood cholesterol levels and function as a tool to prevent and treat cardiovascular

disease (Dunn-Emke *et al.*, 2001). Although studies have reported many cholesterol-lowering agents, certain bacteria can diminish the cholesterol level present in the liquid system (Saavedra *et al.*, 2003) and the blood serum (Hlivak *et al.*, 2005). Currently, cholesterol oxidase (CHO) from bacterial species oxidizes cholesterol and participates in the initial step of cholesterol metabolism (Devi and Kanwar, 2017).

CHO is a flavoenzyme belonging to the oxidoreductase family that catalyzes the oxidation of 3 β -hydroxysteroids, and the isomerization of cholest-5-en-3-one functions as an intermediate to form an end product 4-cholesten-3-one (4-CHN) (Kumari and Kanwar, 2012). Initially, CHO presence was detected in *Rhodococcus erythropolis* (Turfitt, 1946). Later, it was identified in numerous bacteria, including both Gram-positive and negative (Kumari and Kanwar, 2012). Two extracellular CHOs were characterized later, which reacted with cholesterol, and the obtained yield was diagnosed as 4-CHN (Rhee *et al.*, 2002). According to

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Cruz *et al.* (2001), the bioconversion process superficially occurs due to the involvement of an aqueous and organic double-phasic system.

In the pharmaceutical industry, steroids can function as leading compounds among the most extensively used products available in the market. Chemical or microbial routes implicate the synthesis of steroid drugs that involve complex mechanisms. Initially, steroid precursors, a starting material engaged in the transformation of steroid to drug intermediates, were made for the production of the final material. Microbial transformation cleaves the steroid precursor convoluted side chain into an individual step which assimilates effective modification in the steroid nucleus (Rao *et al.*, 2013). The manufacture of steroid drugs and hormones using microorganisms is potentially encouraged in large-scale industrial processes. The biotransformation related to chemical synthesis required sufficient tools for the massive production of natural or diversified steroid drugs. Later, with innumerable therapeutic advantages, such as increased potency, longer half-life in the bloodstream, easy deliverable method and lesser side effects, the natural bioconversion process has been focused on (Bortolini *et al.*, 1997).

Commercially made steroid-based drugs exhibit a wide range of therapeutic applications and represent the maximum marketed products in the pharmaceutical industry after antibiotics. The production of steroidal compounds, such as estrogens, androgens, and progestogens, are commercially synthesized using a combination of microbiological and chemical processes (Fernández-Cabezón *et al.*, 2018). In steroid transformations, 4-CHN is regarded as a precursor for the synthesis of other drug intermediates, such as androst-4-ene-3,17-dione and androsta-1,4-di-ene-3,17-dione, which are leading initial components for the synthesis of anabolic drugs and contraceptive hormones (Dogra and Qazi, 2001).

The synthesis of 4-CHN by chemical methods require many adverse solvents like chloroform, methanol, and benzene. At the same time, these methods take a long time with multistep processes to yield 4-CHN. In order to overcome these, a specific action for the manufacture of 4-CHN using microbes has been widely approached. For better bioconversion, the enzymes involved in the transformation of the steroid compounds have to be improved. Even though many methods are available, metal ions or surfactants are supplied to develop enzyme productivity, optimization of transformation system, and heterologous expression of the CHO gene, and are also applied to boost steroid bioconversion (Wu *et al.*, 2015).

In modern drug discovery, a molecular docking method proves the action of small molecules in the binding site of a target protein. This protein–ligand or protein–protein docking plays a crucial role in predicting the orientation of the ligand where it binds to the receptor (Pagadala *et al.*, 2017). ArgusLab is a new molecular modeling package used for molecular docking study. This produces crystallographic binding orientations. This functions as an effective teaching tool to illustrate molecular docking (Abdelouahab *et al.*, 2008).

In this research, CHO from *Bacillus cereus* strain KAVK5 converts cholesterol into 4-CHN in a double-phasic (i.e., aqueous and organic) system. Optimization of the CHO productivity for the bioconversional process is assessed. Furthermore, produced

4-CHN has been investigated *in silico* molecular docking mode against obesity, diabetes, cancer, and bacterial proteins.

MATERIALS AND METHODS

Isolation and identification of *B. cereus* strain KAVK5

Bacillus cereus strain KAVK5 was isolated from a ghee sample collected in Chennai, Tamil Nadu, India. The ghee was continuously diluted with 9 ml sterile distilled water, further cultured in a cholesterol-incorporated nutrient medium, followed by incubation at 37°C for 24 hours. The strain KAVK5 was primarily resolved based on physical, morphological, and biochemical parameters (Bergey and Holt, 1994). Molecular identification of species was attained by partial amplification of the 16S rRNA gene segment. The amplified gene product was submitted to National Center for Biotechnology Information (NCBI) database, and the accession number was obtained.

Detection of CHO production from KAVK5 using qualitative and quantitative methods of analysis

Qualitative analysis of CHO

The production of CHO from KAVK5 was corroborated by the colony staining method. Progression of red color indication occurred due to quinoneimine dye formation, revealing the evidence of CHO-producing bacterial strains. Furthermore, substantial evidence was affirmed for the synthesis of CHO using the CHO indicator plate method. Strain KAVK5 was cultured on a CHO indicator plate and incubated at 30°C. As a result, the conversion of H₂O₂ turned the medium color into brown, thus substantiating CHO production (Lashkarian *et al.*, 2010).

Quantitative analysis of CHO

CHO productivity from KAVK5 was calibrated according to the method defined by Yang and Zhang (2012). The enzyme produced in the medium was spectrophotometrically analyzed at 500 nm, and their action was calculated, as mentioned in Amutha and Kokila's (2016) study.

Optimization of medium components for the production of CHO

Differently enriched medium components were modified for the production of CHO from KAVK5. In this study, five different media were initially optimized: media 1 (Ahmad *et al.*, 1992); media 2, containing ammonium chloride (1.0 g/l), dipotassium phosphate (0.25 g/l), magnesium sulfate heptahydrate (0.25 g/l), ferrous sulfate heptahydrate (0.001 g/l), yeast extract (5.0 g/l), and steroid-Tween 80 (20 ml at pH 7.0), with the modification of Arima (1969); media 3 (Varma and Nene, 2003); media 4 (Wu *et al.*, 2015); and media 5 (Lee *et al.*, 1997) were designed. The enzymatic assay determined the volume of enzymes produced by the isolates at 12, 24, 32, 48, 64, and 80 hours. Among the five optimized media, medium 1 contains cholesterol (0.1 g/100 ml), glucose (2.0 g/100 ml), yeast extract (0.5 g/100 ml), NH₄NO₃ (0.2 g/100 ml), K₂HPO₄ (0.02 g/100 ml), and MgSO₄·7H₂O (0.03 g/100 ml) (pH 7.0) that produced maximum CHO, which is preferred for further course of action. Bacteria were cultivated at 37°C with shaking at 150 rev minute⁻¹. Later, the production medium was adjusted with different carbon sources (sucrose, fructose, maltose,

glucose, and xylose) at a concentration of 2.0%. Accordingly, nitrogen sources (yeast extract, beef extract, peptone, ammonium chloride, sodium nitrate, and ammonium nitrate) were added at a concentration of 0.5% and 0.2% by replacing yeast extract and ammonium nitrate in the basal medium, respectively. In the medium, CHO production was further enhanced with different metal ions at a concentration of 0.03% (zinc sulfate, manganese sulfate, ferrous sulfate, copper sulfate, and magnesium sulfate). Additionally, pH (6, 6.5, 7.0, 7.5, and 8.0) activity for CHO production was adjusted. Subsequently, the medium was scrutinized by different temperatures (30°C, 40°C, 50°C, 55°C, and 65°C). Finally, the optimized enriched media was explored using various times, such as 12, 24, 32, 48, 64, and 80 hours. The amount of CHO produced by the isolates was proposed by the enzymatic assay. Due to the effect of the components in the media, the fermentation media was decisively designed for the maximum production of CHO.

Purification and molecular weight determination of CHO from KAVK5

The strains of KAVK5 cultured in an optimized enriched medium were obtained and centrifuged at 4°C cooling condition for 15 minutes at 10,000× *g*. The pellet was discarded, and the remaining supernatant was preferred for the purification of the extracellular enzyme. The supernatant was exposed to ammonium sulfate (80% w/v) precipitation, followed by dialysis against 0.05 M sodium phosphate buffer maintained at pH 7.0 overnight. The protein precipitated within the dialyzed membrane and was immersed in the sodium phosphate buffer (0.05 M, pH 8.0). The protein substances were loaded on to the Diethylaminoethyl cellulose ion-exchange column pack and washed using a buffer. The collected precipitated protein was loaded, and the eluted sample was collected by adding 0.1–0.5 M NaCl in the sodium phosphate buffer. For each molarity, 10 fractions were collected; further CHO activity was explored at every fraction. At the end of the experiment, the fraction containing CHO was pooled out and the purified CHO molecular weight was resolved by the SDS-PAGE method (Laemmli, 1970). The purified fraction containing CHO was run on 12% gel along with the standard marker. The molecular weight was inspected by comparing the protein band with a standard marker.

Enzymatic bioconversion of cholesterol to 4-CHN using CHO

Biological modification of cholesterol to 4-CHN using CHO from KAVK5 was implemented in an aqueous/organic biphasic system. The conventional condition was maintained as follows: 1 g of cholesterol with 130 ml of aqueous and isoamyl alcohol organic mixture (10:3 v/v) in a 500 ml flask; the content was kept at 250× *g*, 30°C at 3 hours, and subsequently oxygen was provided (Liu *et al.*, 1996).

Extraction and purification of 4-CHN from the bioconversion system

After the bioconversion process was completed, 4-CHN and other cholesterol derivatives were retrieved with an equal volume of ethyl acetate; subsequently, the content was treated with water to eliminate the enzyme. The organic layer was separated from the extract and condensed under a vacuum. The recovered product was loaded on silica gel (300–400 mesh) packed columns

and washed with petroleum ether. The column was washed with solvents like ethyl acetate and petroleum ether in the ratio of 1:40 to 1:20 v/v to conduct gradient elution. The eluent in the column was pooled out in a 10 mL test tube and condensed in a rotary evaporator to obtain the final product of 4-CHN (Wu *et al.*, 2015). Thin-layer chromatography was carried out to detect the presence of a derivative product from KAVK5. The silica gel plate was coated, and the eluent was spotted. These TLC plates were placed in a chromatography tank containing 1.5 ml of hexane with 0.5 mL of ethyl acetate. After drying, the plates were treated with iodine vapor until the appearance of spots in the plates.

Molecular docking studies of 4-CHN

In silico molecular docking studies were carried out to predict the mode of action of the ligand 4-CHN against proteins, such as pancreatic lipase (PL), alpha-ketoglutarate-dependent dioxygenase (FTO), sterol regulatory element-binding protein 1, fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC) (obesity proteins), α -amylase (AMS), α -glucosidase (GLS), glycogen synthase kinase-3-alpha (GSK1), glycogen synthase kinase-3-beta (GSK2) (diabetes proteins); cyclin-dependent kinase-2 (CDK-2), human topoisomerase IIa (HT), vascular endothelial growth factor receptor-2 (VEGFR-2) (cancerous proteins), dihydropterotate synthase (2VEG), and DNA gyrase subunit B (3TTZ) (bacterial proteins).

Ligand preparation

4-CHN was selected as the ligand molecule. The structure was constructed using the ChemSketch tool and then converted into a 3D structure.

Receptor protein preparation

The Protein Data Bank (PDB), a comprehensive portal, was used to retrieve the 3D structure of large biological macromolecules, including proteins and nucleic acid. The structure of the proteins, such as PL (ID: 1GPL), FTO (ID: 4IE0), SREBP1 (ID: 1AM9), FAS (ID: 2JFD), ACC (ID: 2YL2), AMS (ID: 1C8Q), GLS (ID: 5KZW), GSK1 (ID: 2DFM), GSK2 (ID: 1GNG), CDK-2 (ID: 1AQ1), HT (ID: 4FM9), VEGFR-2 (ID: 1VR2), 2VEG (ID: 2VEG), and 3TTZ (ID: 3TTZ), were downloaded from PDBSum database. RasMol tool was used to visualize the 3D structure of proteins.

ArgusLab tool used for molecular docking studies and visualization of docked substances

ArgusLab is a molecular docking tool employed to generate a grid, calculate dock score, and assess the interaction between the inhibitors with the active site of targeted enzymes proteins. According to Tanguenyongwatana and Jongkon (2016), ArgusLab, a user-friendly program, affords quick calculation. It has an accessible user interface in molecular docking studies. ArgusLab 4.0.1 functions with grid resolution of 0.40 Å. Energy calculations appraised the stability of the docked structure and the number of hydrogen bonds formed (Thompson, 2004). The most satisfying binding configuration was developed based on the hydrogen bond interactions between 4-CHN and the binding site of proteins. PyMol software demonstrated molecular models and created publication-quality images. The final docked structures

were then visualized using PyMol viewer, and the docking results were predicted.

Statistical analysis

In this report, the entire investigations were carried out in triplicate, and the results were revealed as mean \pm standard deviation (SD). Using Duncan's multiple range test (DMRT), significance differences were determined and $P < 0.05$ values were statistically significant. This data was provided using SPSS statistical version 16.0.

RESULTS AND DISCUSSION

Cholesterol is a fatty substance that abundantly grows in all the cells of the body. Accumulation of cholesterol deals with multiple disorders, includes stroke, coronary heart disease, peripheral vascular disease, high blood pressure, and diabetes (Pekkanen *et al.*, 1990). Cholesterol-degrading bacteria can be discriminated from food samples such as yoghurt, whey, milk, cheese, and raw milk (Yehia *et al.*, 2015). A few bacterial species not only absorb cholesterol as a carbon source (Kovalenko *et al.*, 2004), but they also decompose using CHO enzyme and produce multiple intermediate products (Liu and Shan, 2006). However, in this report, *B. cereus* strain KAVK5 produced CHO and was extensively applied for biological modification into 4-CHN.

Identification of strain KAVK5

The cholesterol-lowering *B. cereus* strain KAVK5 was isolated from the collected ghee samples and determined according to morphological and biochemical characteristics. *Bacillus subtilis* SFF34 from the Korean traditional fermented flatfish degraded cholesterol at 0.2% in the medium (Kim *et al.*, 2002). Accordingly, *Pseudomonas* sp., *Bacillus* sp., and *Streptomyces* sp. isolated from the soap and vegetable oil industrial wastes were potentially degraded cholesterol (Saranya *et al.*, 2014). In this report, strain KAVK5 was Gram-positive and also exhibited a positive effect on catalase and oxidase test. Dash *et al.* (2015) identified *B. subtilis* BI19 molecularly by sequencing the 16S rDNA gene region. Molecular-level identification was crucial to confirm the organisms at the species level; consequently, the 16S rRNA gene region was targeted to perform amplification, followed by sequencing. The obtained amplified data were submitted to NCBI and the accession number obtained was KP792776, which stated the organism as *B. cereus* and named the strain KAVK5 (Fig. 1). The genus *Bacillus* was treated as beneficial bacteria for the production of extracellular enzymes. Majority (60%) of the commercially producing enzymes were obtained from *Bacillus* species (Burhan *et al.*, 2003).

Qualitative and quantitative analyses of CHO from strain KAVK5

CHO harvested from KAVK5 was executed using qualitatively and quantitative methods. A red color pattern consummate around the bacterial colonies was detected in the colony staining test (Fig. 2A). Subsequently, the indicator plate method also strongly verified the production of CHO, which could transform the medium color into brown. The color formation revealed in the medium was due to azo components, thus leading to H₂O₂ generation (Fig. 2B). CHO production was scrutinized in

Serratia marcescens W1 and *Bacillus pumilus* W8. The red and brown colors' progression was well documented in both the colony staining method and the indicator plate assay (Wali *et al.*, 2019). CHO is a commercially produced enzyme used to determine cholesterol found in the food, serum, and other clinical samples. *Rhodococcus* sp. from urban compost and dairy soil samples produced CHO. Maximum yield was enforced by optimizing the production medium using temperature, pH, carbon, and nitrogen sources (Kaur *et al.*, 2015).

Furthermore, in this study, after the confirmation of CHO production in a qualitative method, the volume (U) of enzyme production was quantified using spectrophotometry. In this regard, five designed media components were acquired; one of the media exhibited maximal production of 0.86 U/mL. Later, this medium component could be embraced for further modification to enhance CHO production (Fig. 2C). In this research, the yield of CHO from KAVK5 had been expanded because of the large-scale formulation; accordingly, optimization of the fermentation medium [carbon, nitrogen (organic and inorganic), metal ion, pH, temperature, and incubation time] was majorly noted.

Optimization of CHO production and purification

In the microbial fermentation process, carbon sources function as a major component responsible for cellular substance synthesis to provide energy widely (Devi and Kanwar, 2017). Accordingly, Yazdi *et al.* (2001) revealed the beneficial effects of various steroids and sugars enacted as a carbon source on CHO production using *Rhodococcus equi* 2C. So, their findings proved that cholesterol was selected as the prime carbon source. Consequently, the carbon source in the medium was altered; in that matter, xylose displayed the greatest production of 1.19 U/mL (Fig. 3A), whereas, in the organic and inorganic nitrogen source of adjustment, peptone and sodium nitrate provide CHO (1.24 and 1.39 U/ml, respectively) (Fig. 3B). The utilization of nitrogen sources in the medium was an essential factor for the production of CHO. Many researchers had a high impact on organic and inorganic nitrogen sources. Of all the data, ammonium and yeast extract had more influence on CHO productivity (Devi and Kanwar, 2017). Modifying the medium using metal ion sources indicates that manganese sulfate yields 1.47 U/ml (Fig. 3C). pH has a great response to the productivity and synthesis of enzymes. CHO actively works best at a pH of 6.5–8.0 (Doukyu, 2009). Later, the yield was increased by about 1.68 U/ml, while adjusting the optimal pH at 7.0 (Fig. 3D) and, simultaneously, the production rate gradually increased up to 1.81 U/ml at 30°C (Fig. 3E). Different cultivation temperatures (34.2°C–39.8°C) were regulated for CHO production, and the result stated that the cell reached maximum growth for all the temperatures assessed (Devi and Kanwar, 2017). However, optimum growth was obtained at a lower temperature range of 35°C, with a maximum of 37°C. Moreover, cell growth was obtained for CHO production at 39°C (Lee *et al.*, 1999). The enriched medium exhibited tremendous output, which was designed for further study. KAVK5 showed the highest productivity (1.86 U/ml) at 32 h of the incubation (Fig. 3F). The designed enriched medium used for the production of CHO from KAVK5 consisted of the following components: cholesterol (0.1 g.ml⁻¹), xylose (2.0 g.ml⁻¹), peptone (0.5 g.ml⁻¹),

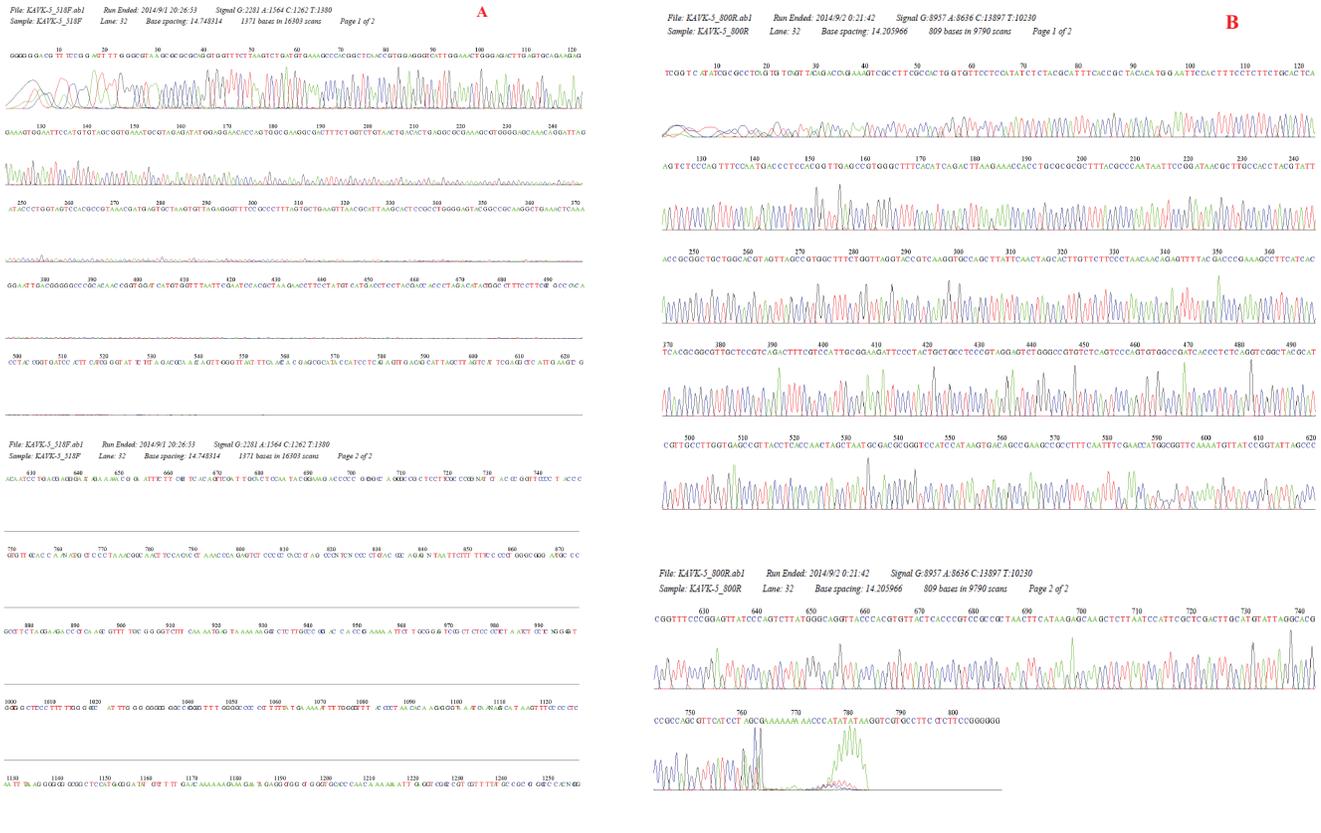


Figure 1. 16S rRNA gene amplification of *B. cereus* strain KAVK5. (A) Forward chromatogram of *B. cereus* strain KAVK5. (B) Reverse chromatogram of *B. cereus* strain KAVK5. (C) NCBI data on the submitted gene sequence of *B. cereus* strain KAVK5.

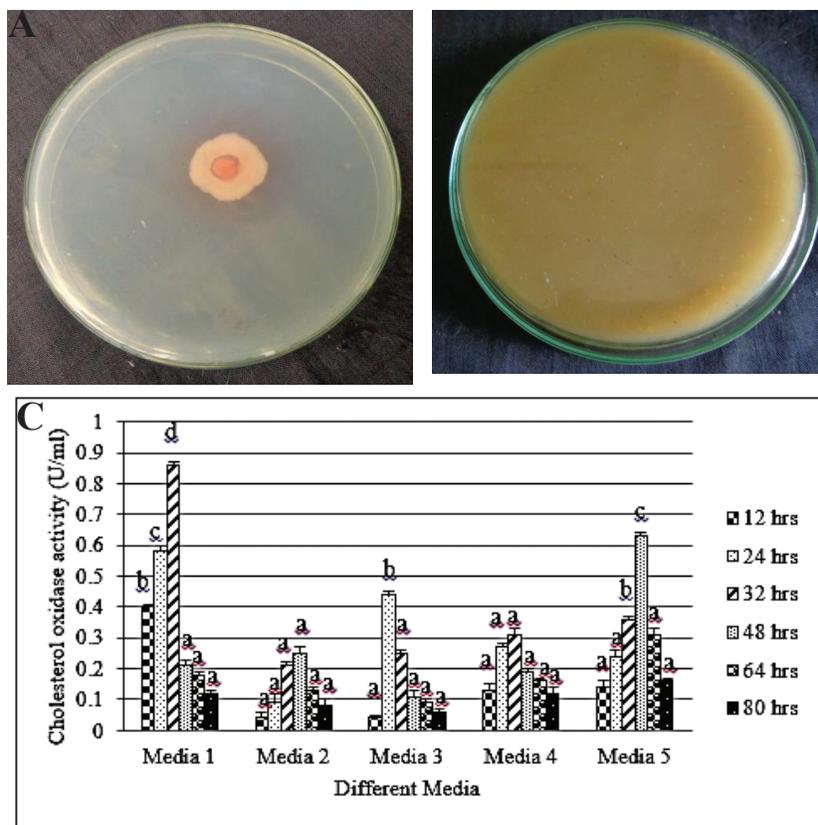


Figure 2. (A) Colony staining method of *B. cereus* strain KAVK5. (B) Cholesterol oxidase indicator plate of *B. cereus* strain KAVK5. (C) Different media for the production of CHO from *B. cereus* strain KAVK5. Values are expressed as mean \pm standard deviation for three samples ($n = 3$) in each group. Superscripts in the column show significant differences at the $p < 0.05$ level. Analysis of variance (ANOVA) was followed by DMRT. Comparison is made between media 1 and 5.

sodium nitrate (0.2 g.ml^{-1}), and manganese sulfate (0.03 g.ml^{-1}). pH 7.0 was maintained at 30°C and incubated at 32 hours.

CHO was purified using differential ammonium sulfate precipitation, ion exchange, and gel filtration chromatography (Kumari and Kanwar, 2012). *Bacillus subtilis* from tiger excreta produced CHO, and purification was additionally processed by salting out the CHO using ammonium sulfate, followed by dialysis and riboflavin-affinity chromatography. This enzyme exhibited a molecular weight of about 105 kDa (Kumari and Kanwar, 2016). *Bacillus subtilis* YS01 produced CHO of approximately 56 kDa, which was widely filtered by ammonium sulfate precipitation, dialysis, DEAE cellulose ion-exchange chromatography, and Sephadex G-100 gel filtration (Aruna *et al.*, 2017). In addition, CHO in the enriched medium was purified with the help of DEAE cellulose ion-exchange column chromatography. The purified CHO revealed its molecular weight at 32.8 kDa in the SDS-PAGE method (Fig. 4).

Production and purification of 4-CHN using enzymatic bioconversion method

The effect of the organic solvent, like isoamyl alcohol, used for the bioconversion study was examined in an organic/

aqueous system. The conversion rate was higher in the aqueous/organic biphasic system, comparable to the simple aqueous phase (Wu *et al.*, 2015).

Experimental condition

Different cholesterol concentrations were supplied to the bioconversion medium containing 100 mL of enzyme solution with 30 ml of organic solvent during the experimental period. Increasing the concentration of cholesterol decreased or delayed the bioconversion process, thus ultimately resulting in enzyme deactivation. Accordingly, 1 g of cholesterol was fixed up to achieve the conversional step, increasing the bioconversion rate. The biological conversion of cholesterol to 4-CHN produced H_2O_2 , which functioned as a strong oxidant resulting in the denaturation of CHO protein (Wu *et al.*, 2015).

In this study, the fermentation procedure was processed in a 0.5 l flask containing 30 ml of isoamyl alcohol, 100 ml of CHO from KAVK5 strain solution, and 1 g of cholesterol maintained at 30°C for 30 minutes at $250\times g$. After the bioconversion condition was completed, an equal amount of ethyl acetate was added to the component, and the organic layer was separated, followed by condensation. The residual substances were dropped onto a

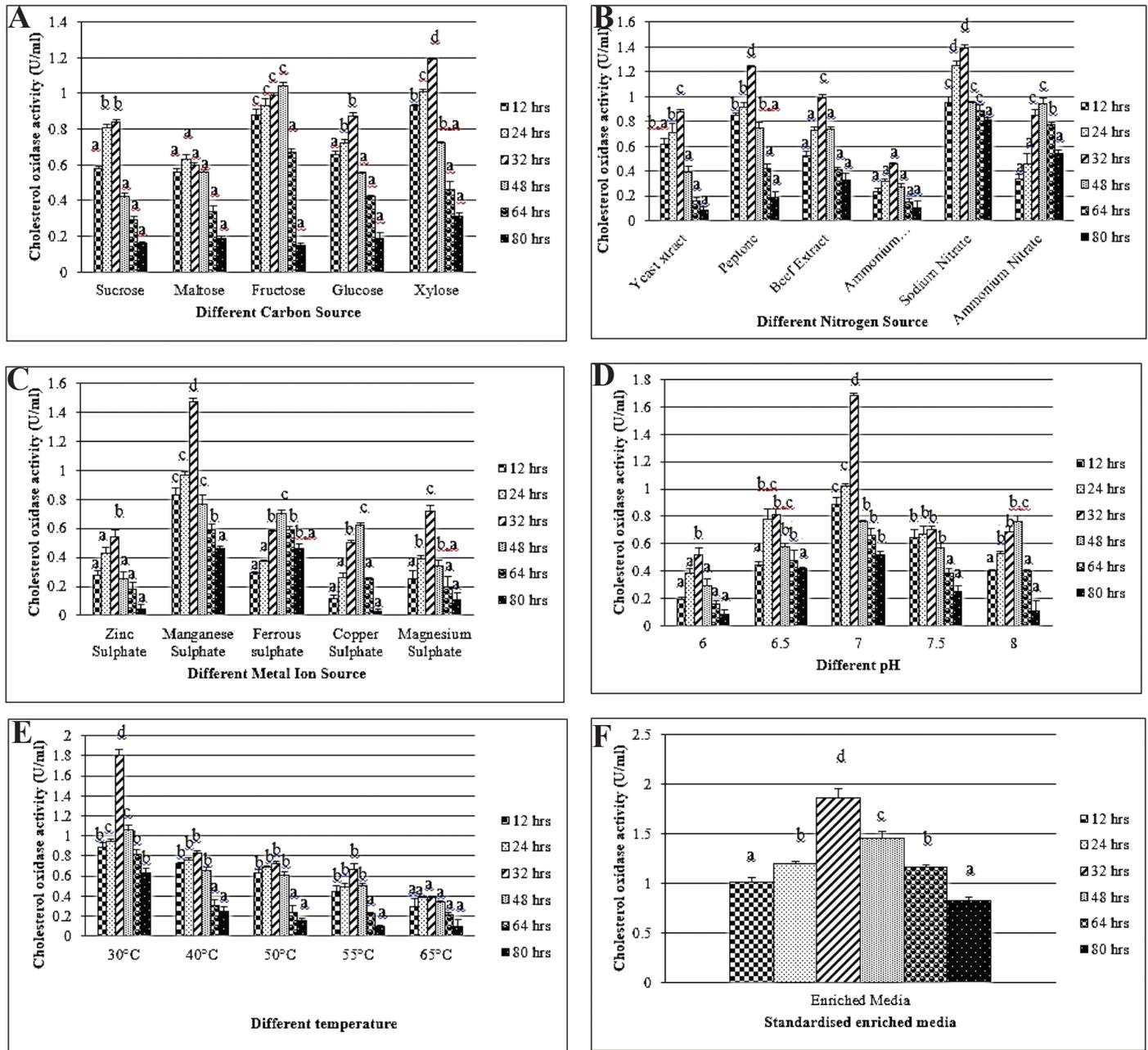


Figure 3. (A) Different carbon sources for the production of CHO from *B. cereus* strain KAVK5. Values are expressed as mean \pm standard deviation for three samples ($n=3$) in each group. Superscripts in the column show significant differences at the $p < 0.05$ level. ANOVA was followed by DMRT. Comparison is made between carbon sources. (B) Different nitrogen sources for the production of CHO from *B. cereus* strain KAVK5. Values are expressed as mean \pm standard deviation for three samples ($n=3$) in each group. Superscripts in the column show significant differences at the $p < 0.05$ level. ANOVA was followed by DMRT. Comparison is made between nitrogen sources. (C) Different metal ion sources for the production of CHO from *B. cereus* strain KAVK5. Values are expressed as mean \pm standard deviation for three samples ($n=3$) in each group. Superscripts in the column show significant differences at the $p < 0.05$ level. ANOVA was followed by DMRT. Comparison is made between metal ion sources. (D) Different pH for the production of CHO from *B. cereus* strain KAVK5. Values are expressed as mean \pm standard deviation for three samples ($n=3$) in each group. Superscripts in the column show significant differences at the $p < 0.05$ level. ANOVA was followed by DMRT. Comparison is made between pH. (E) Different temperatures for the production of CHO from *B. cereus* strain KAVK5. Values are expressed as mean \pm standard deviation for three samples ($n=3$) in each group. Superscripts in the column show significant differences at the $p < 0.05$ level. ANOVA was followed by DMRT. Comparison is made between temperatures. (F) Different incubation times for the production of CHO from *B. cereus* strain KAVK5. Values are expressed as mean \pm standard deviation for three samples ($n=3$) in each group. Superscripts in the column show significant differences at the $p < 0.05$ level. ANOVA was followed by DMRT.

column chromatography (300–400 silica mesh), and 4-CHN was collected and subjected to thin-layer chromatography. The end product was collected and condensed under a vacuum evaporator, and the obtained yield was calculated, reaching a yield of about

1.23 g for every bioconversion step (Fig. 5). Researchers reported that the bioconversion process was achieved depending on the three main actions: mechanism of enzyme catalysis, metabolic pathway, and finally optimization of enzyme activity (Wu *et al.*,

2015). According to them, petroleum ether used with enzyme solution increased its yield up to $4 \text{ g l}^{-1} \text{ h}^{-1}$, corresponding to the conversional rate at 90%. Shao *et al.* (2015) produced 4-CHN of about 0.61 g l^{-1} and 0.75 g l^{-1} with the conversional rates of 67% and 83% using genetically modified cells, respectively. The obtained product was spotted with standard 4-CHN on a silica gel plate and placed in a chromatography tank. Normal cholesterol's retardation factor (Rf) exhibited a 0.5 value, and the standard 4-CHN revealed a 0.6 value. The purified compound in this study reported around 0.63 value.

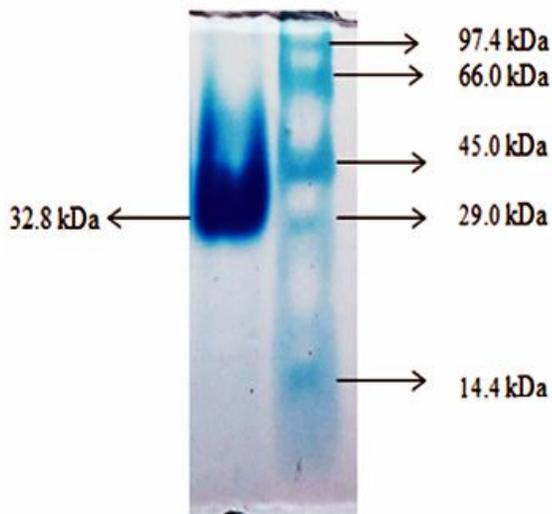


Figure 4: Molecular weight determination of CHO from *B. cereus* strain KAVK5.

Molecular docking studies of 4-CHN against proteins

The molecular docking study is a fast-growing area to interpret and predict the interaction between a ligand and a receptor protein macromolecule. This report targeted the development of an active drug against obesity, diabetes, cancer, and bactericidal effect. We selected 4-CHN as a lead compound for the drug design. The molecular structure of 4-CHN used in this study was drawn using ACD/ChemSketch (Fig. 6A), and the 3D structure of compounds is shown in Fig. 6B.

Interaction between 4-CHN with obesity-associated proteins

Obesity is a metabolic syndrome defined by extreme weight gain to height distribution primarily responsible for expanded fat deposition, associated with a larger calorie absorption related to the energy output (Mohapatra *et al.*, 2015). Pancreatic lipase, a key enzyme, plays an active role in breaking dietary fats by hydrolysing the fat molecule into mono-acylglycerol, and fatty acids diminish the intake of triglycerides (Birari and Bhutani, 2007). In an anti-obesity study, this enzyme was widely focused on developing natural products (Sugiyama *et al.*, 2007). According to Gurudeeban *et al.* (2012), the FTO gene interacted with obesity, which is considered a health risk factor around the world. The occurrence of obesity is due to the enlargement of fat mass, resulting from excess adipose cell size and number in the cytoplasm of adipocytes which participates in enzymes such as fatty acid synthase (Devlin *et al.*, 2000). Inhibition of adipose tissue enlargement was considered as a target to control obesity disorder. This process can be regulated by transcriptional factors like sterol regulatory element-binding protein 1 (SREBP1) (Park *et al.*, 2019). Acetyl-CoA carboxylase played a vital role in fatty acid metabolism and was designed as a potent target for developing a new therapeutic agent in controlling obesity (Gago *et al.*, 2011). In the current study, PL, FTO, SREBP1, FAS, and ACC proteins codes for obesity disorder were docked against the

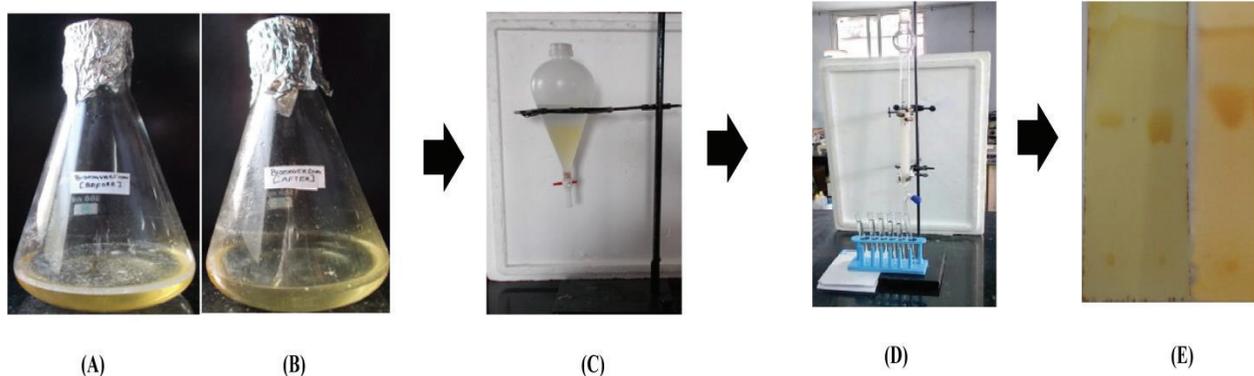


Figure 5. Schematic representation of the bioconversion pathway. (A) Before the bioconversional process. (B) After the bioconversional process. (C) Separation of the organic layer. (D) Column chromatography for the separation of 4-CHN. (E) Thin-layer chromatography: (a) standard 4-CHN; (b) standard cholesterol; and (c) tested 4-CHN.

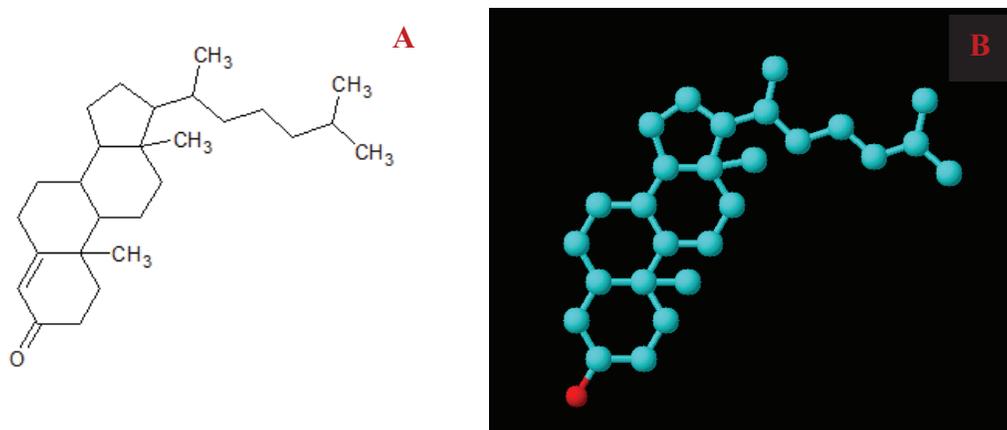


Figure 6. Structure elucidation of 4-CHN. (A) 2-dimensional structure and (B) 3-dimensional structure.

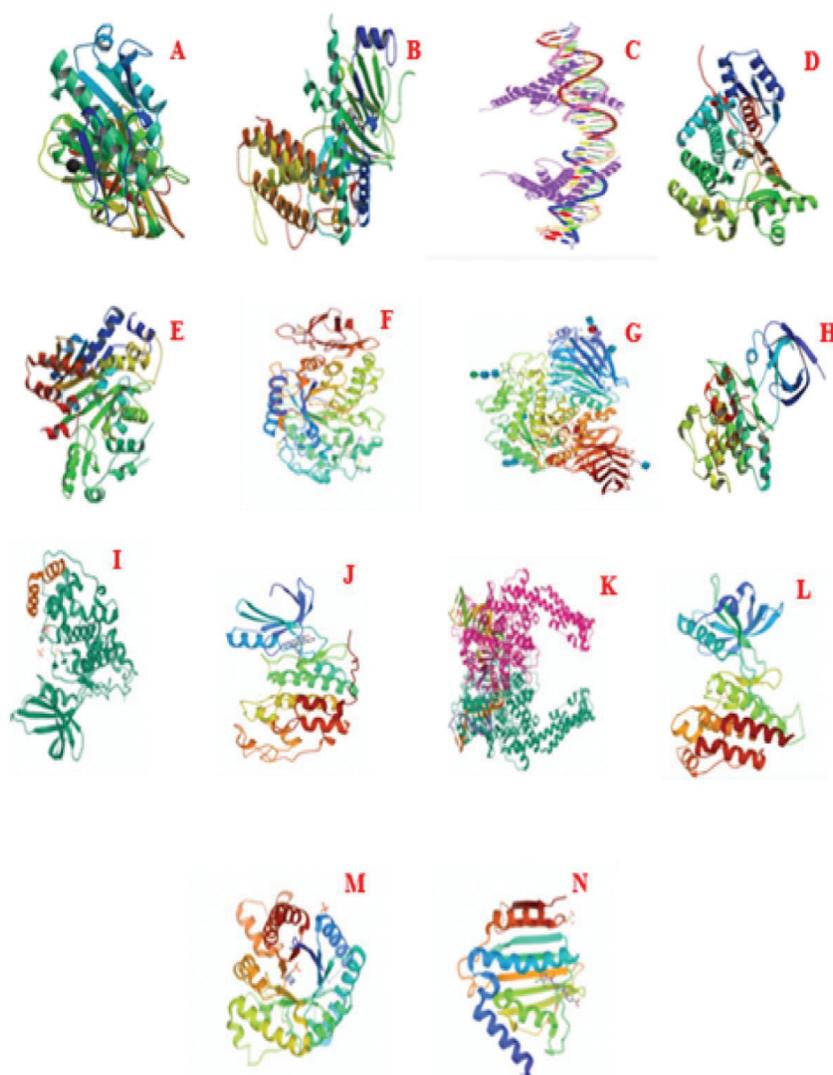


Figure 7. Structure of proteins. (A) Pancreatic lipase. (B) Alpha-ketoglutarate-dependent dioxygenase. (C) Sterol regulatory element-binding protein 1. (D) Fatty acid synthase. (E) Acetyl-CoA carboxylase. (F) α -Amylase. (G) α -Glucosidase. (H) Glycogen synthase kinase-3-alpha. (I) Glycogen synthase kinase-3-beta. (J) Cyclin-dependent kinase-2. (K) Human topoisomerase IIa. (L) Vascular endothelial growth factor receptor-2. (M) Dihydropteroate synthase. (N) DNA gyrase subunit B.

Table 1. Docking energy of the docked complexes from ArgusLab software.

Proteins	Clustering and re-clustering the final poses (final unique configuration)	Best ligand pose energy (kcal/mol)	Docking run elapsed time (seconds)
A	117	-14.5252	48
B	124	-13.3941	67
C	132	-11.4437	16
D	119	-12.8343	55
E	137	-12.8518	77
F	120	-12.874	68
G	98	-12.3602	81
H	122	-13.9566	62
I	134	-12.6885	60
J	126	-13.6546	35
K	129	-12.3318	87
L	120	-12.789	38
M	137	-16.1199	15
N	122	-10.953	9

A) pancreatic lipase; B) alpha-ketoglutarate-dependent dioxygenase; C) sterol regulatory element-binding protein 1; D) fatty acid synthase; E) acetyl-CoA carboxylase; F) α -amylase; G) α -glucosidase; H) glycogen synthase kinase-3-alpha; I) glycogen synthase kinase-3-beta; J) cyclin-dependent kinase-2; K) human topoisomerase IIa; L) vascular endothelial growth factor receptor-2; M) dihydropteroate synthase; and N) DNA gyrase subunit B.

ligand 4-CHN, and their proteins were downloaded from the PDB database, which is shown in Fig. 7(A–E).

Interaction between 4-CHN with diabetes-associated proteins

α -Amylase, an enzyme also called endonuclease enzyme, is engaged in the digestion of polysaccharides, whereas α -glucosidase is situated in the intestinal part which participates in absorbing oligosaccharides (Telagari and Hullatti, 2015). These enzymes were treated as key enzymes that maintained blood glucose levels by slowing the digestive process and absorption of carbohydrates and incertins (Okechukwu *et al.*, 2020). Type 2 diabetes mellitus appears more than that of the total diabetic population, which correlates with obesity-induced insulin resistance and subsequently secreted insulin from the β -cells of the pancreas. Glycogen metabolism plays an essential role in maintaining glucose homeostasis and is regulated by two enzymes like glycogen synthase and phosphorylase. Glycogen synthase kinase (GSK) is a negative regulator of the insulin signaling pathway composed of two similarly linked isoforms, namely GSK-3 α and GSK-3 β (Devi *et al.*, 2018). Consequently, AMS, GLS, GSK1, and GSK2 were intended for docking against 4-CHN, and the respective proteins were downloaded from the PDB database (Fig. 7F–I).

Interaction between 4-CHN with cancer-associated proteins

Numerous types of enzymes could develop cancer. Among those, CDK-2, HT-I α , and VEGFR-2 were considered the most common enzymes actively involved in the development of cancerous cells (Sarkar *et al.*, 2021). Accordingly, CDK-2,

HT, and VEGFR-2 were targeted to achieve docking against the ligand, and the correspondent proteins were downloaded from the PDB database (Fig. 7J–L).

Interaction between 4-CHN with associated bacterial proteins

Recently, a difficulty faced by the researchers was the discovery of antimicrobial drugs and other therapies. Bacteria exhibited resistance to the drugs; this occurred due to the establishment of persister cells or biofilms (Puiu *et al.*, 2017). On that account, the biological role of 4-CHN was ensured for antibacterial activity. Drugs potentially target the folate pathway by inhibiting the dihydropteroate synthase enzyme. Moreover, this activity was extensively required for bacterial and synthetic pathways, which also function as a target of interest for antibacterial agents (Potshangbam *et al.*, 2020). DNA gyrase subunit B from bacteria was selected for the *in silico* docking study against ligand anthraquinones, proving antibacterial effect (Arevalo and Amorim, 2021). Based on this, 2VEG and 3TTZ were focused on for docking study against the ligand 4-CHN and the bacterial proteins were downloaded from the PDB database (Fig. 7M and N). This can be strongly proven as the best antibacterial agent.

In this docking study, a stable complex could be found upon the preferred orientation of one molecule (protein) bound to other molecules (ligand). This can be applied using ArgusLab software. The binding action plays a vital part in recognizing docking energy and stability of the receptor–ligand complex, which was authorized by hydrogen bonds. Interaction between the complex molecules produced an energy score, which is presented in Table 1, and the docked complex was visualized

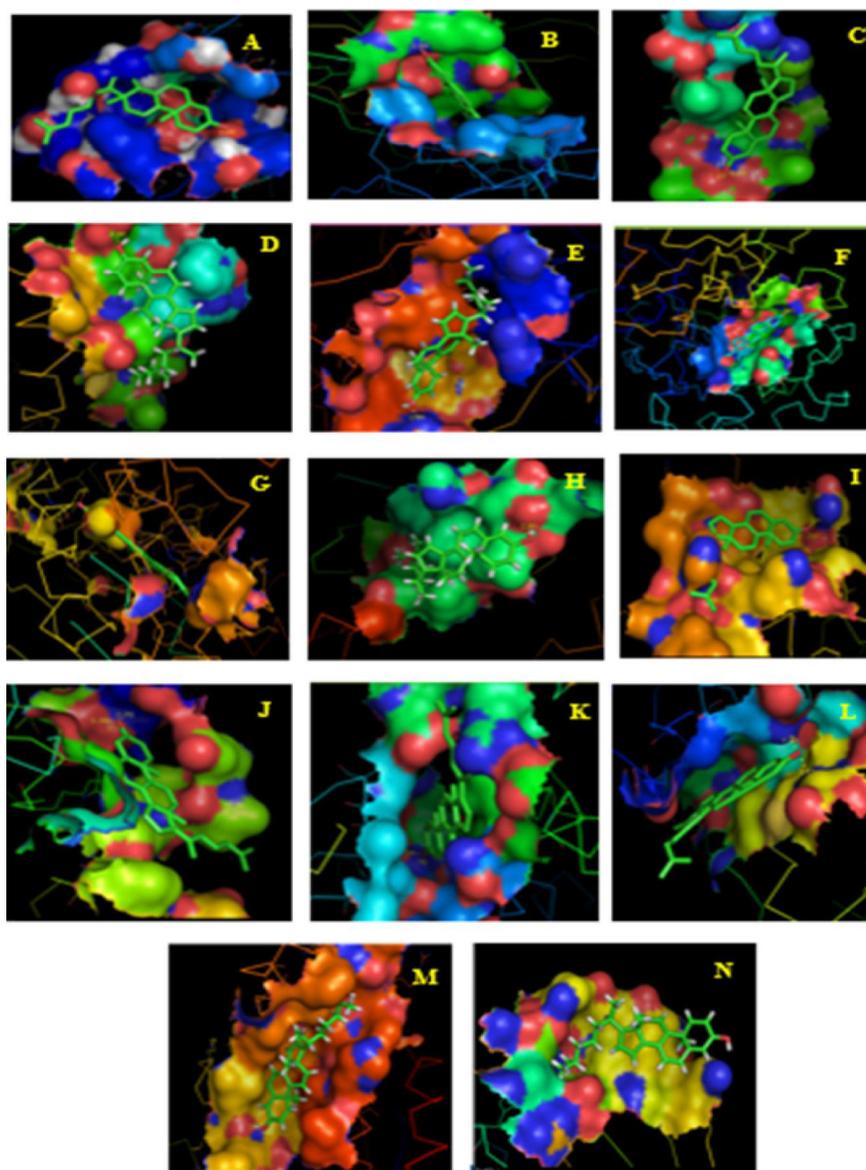


Figure 8. Final docked conformations of 4-CHN with (A) pancreatic lipase; (B) alpha-ketoglutarate-dependent dioxygenase; (C) sterol regulatory element-binding protein 1; (D) fatty acid synthase; (E) acetyl-CoA carboxylase; (F) α -amylase; (G) α -glucosidase; (H) glycogen synthase kinase-3-alpha; (I) glycogen synthase kinase-3-beta; (J) cyclin-dependent kinase-2; (K) human topoisomerase IIa; (L) vascular endothelial growth factor receptor-2; (M) dihydropteroate synthase; and (N) DNA gyrase subunit B.

using PyMol software (Fig. 8). The interaction of docked complex produces binding residues, atoms, number of hydrogen bonds, and distances; the docking scores are listed in Table 2. The table also displays the amino acids in the active region of studied proteins interacting with 4-CHN.

4-CHN interaction with proteins by the *in silico* method calculated the minimum binding energy (kcal/mol). In a molecular docking analysis, the parameters considered essential tools include hydrogen bond interactions, n-n interactions, binding energy, active

site residues, and orientation of the phytochemicals with the receptors (Madeswaran *et al.*, 2011). This result shows that there was a presence of a binding site between the protein and ligand. The docking was also valid by the development of a hydrogen bond between them. From the above docking result, 4-CHN docked well to these proteins responsible for obesity, diabetes, cancer, and bactericidal effect. The result of the Lipinski rule suggests that the analyzed compound acts as the best therapeutic pharmaceutical drug.

Table 2. Docking score of 4-CHN with diseased proteins.

Ligand	Diseases	Proteins	Residue	Distance (Å)	Docking Score (Kcal/Mol)	No. of H-Bonds	
4-Cholesten-3-one	Obesity	Pancreatic lipase	THR 36	2.09	-14.52	1	
		Alpha-ketoglutarate-dependent dioxygenase	LYS 216	2.00	-13.39	1	
		Sterol regulatory element-binding protein 1	THR 353	1.97			
			LYS 347	2.58	-11.44	3	
			ALA 355	3.48			
			GLU 597	2.90	-12.83	1	
		Fatty acid synthase	GLU 597	2.90	-12.83	1	
		Acetyl-CoA carboxylase	ARG 539	2.89	-12.85	1	
		α -amylase	LYS 200	2.54	-12.87	1	
		α -glucosidase	SER 627	3.09	-12.36	1	
	Diabetes	Glycogen synthase kinase-3-alpha	ALA 115	2.90	-13.95	1	
		Glycogen synthase kinase-3-beta	GLN 254	3.01	-12.68	2	
			PRO 255	2.87			
			ASP 145	3.30			
	Cancer	Cyclin-dependent kinase-2	THR 14	2.78	-13.65	3	
			THR 14	2.70			
		Human topoisomerase IIa	TYR 590	3.14	-12.33	2	
			GLN 542	1.96			
			Vascular endothelial growth factor receptor-2	LYS 868	2.75	-12.78	2
				ASP 1046	3.17		
Bactericidal effect	Dihydropteroate synthase	LEU 214	1.83	-16.11	1		
	DNA gyrase subunit B	No residue formed	-	-10.95	No Interaction		

CONCLUSION

Bacillus cereus strain KAVK5 produced economically important CHO. The productivity was enhanced by the optimization of the fermentation medium, resulting in an improved bioconversion process. In a biotransformation system, this enzyme collaboratively provides a straightforward and reliable conversion of cholesterol into 4-CHN in an aqueous and organic double-phasic system. It was achievable to develop the superior quality of 4-CHN on an extensive range of processes and thus function as a pharmaceutical drug that could be predicted using an *in silico* docking model. 4-CHN is an active metabolite that acts as a ligand for various diseases. In this regard, this active molecule exhibits promising anti-obesity, antidiabetic, anticancer, and bactericidal disorder. However, based on our results, we can state that 4-CHN was likely bound to the protein and thus the interaction primarily recognized that this compound had a positive effect on the tested disorder. The research in this report concludes that the enzymatic production of 4-CHN provides a platform for developing steroid drugs in the pharmaceutical industry.

CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of

data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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