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Application of *Bacillus* sp. COX-T_3 Cholesterol Oxidase in Reduction of Serum Cholesterol and Body Weight Management in a Murine Model

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Abstract: To examine the effect of purified cholesterol oxidase (COX) of a *Bacillus* sp. COX-T_3 for treating high-lipid diet-induced hypercholesterolemia, an experimental mouse model was established. The enzyme was purified by using a Riboflavin-Sephadex G-25 affinity column. After keeping mice assigned to Group 1 to 5 on a high fat diet, the mice showed 14.4, 20.6, 24.4, 19.4 and 17.0 % body weight gain, respectively (mean body weight gain of ~19.0 %) over 9 weeks of intake of high-fat feed. The maximum body weight was attained in the mice assigned to Group 3 (32.73 ± 2.11 g) in comparison to the control regimen kept on normal diet for the same period of time. The results reflected the successful establishment/development of a near obese mouse model by intake of high-fat diet for 9 weeks. The cholesterol level in the high-fat diet fed mice significantly increased with age up to 6th week. For the Group 1 to 5, the high fat diet caused a significant increase of serum cholesterol. The purified COX was intra-muscularly administered to the mice at 0.1 to 0.5 U/dose (100 µl) on alternate days for 5 weeks. The mice assigned to treatment group(s) 3, 4 and 5 showed 2.2, 2.2 and 3.5 % decline in their average body weight(s) that indicated a dose/ concentration dependent effect of administration of *Bacillus* sp. COX-T_3 in bringing a reduction in the body weight(s) of these mice. The reduction of serum cholesterol level by 45.3, 40.9, 22.4, 28.4 and 40.7 % in Group 1 to 5, respectively, was achieved in comparison to the serum cholesterol levels recorded after 9 weeks of intake of high fat diet in mice. Interestingly, the addition of the bacterial COX at 1.2 U.ml^{-1} concentrations to the Hep2C cells slightly promoted the cell viability while the COX at 1.8 U.ml^{-1} exerted a cytotoxic effect on the cell growth as reflected by ~10.6 % decline in the cell viability recorded in MTT assay. The observed data indicated a successful therapeutic application of the purified COX of *Bacillus* sp. COX-T_3 in treating an obese hypercholesterolemic murine model over a reasonable period of enzymatic treatment.

Key words: Cholesterol oxidase; extracellular enzyme; purification; hypercholesterolemic; murine model; weight loss.

Introduction

Traditionally the cholesterol oxidase (COX) enzyme has been used in the detection of serum cholesterol, fabrication of cholesterol biosensor and transformation of sterols. A fast-growing strain of *Mycobacterium* sp. VKM Ac-1815D capable of effective oxidization of sterols (phytosterol, cholesterol and ergosterol) to androstenedione and other valuable 3-oxo-steroids has been reported [5]. The COX efficiently oxidized β-sitosterol, dehydroepiandrosterone, ergosterol, pregnenolone and lithocholic acid. The COX is not a critical enzyme responsible for modification of 3β-hydroxy-5-ene- to 3-keto-4-ene steroids in *Mycobacterium* sp. VKM Ac-1815D [5]. The results validated the statistical approach to find out a potential technique for achieving large-scale production of cell-bound COX from *Rhodococcus* sp. NCIM 2891 [1]. Another important application for which the use of COX alone or along with

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other additives has been suggested is treatment of cholesterolemia. With obesity in human beings becoming a major epidemic and a health challenge, an appropriate animal system is needed to prove the efficacy and safety of employing microbial COX in the treatment of experimental hypercholesterolemia and body weight management. Although the commercial grade cholesterol oxidase(s) are available but the cost prohibits their use as therapeutic tools. High serum cholesterol is a risk factor for cardiovascular disease (CVD). Currently, in addition to a low-fat diet, HMG-CoA reductase inhibitors ("statins") are recommended as first-line treatments to decrease low-density lipoprotein C (LDL-C) level in the blood. About 5-10 % of patients cannot tolerate statin therapy; and oral administration to a patient, wherein the pharmaceutical composition includes an effective amount of cholesterol oxidase, optionally at least one second therapeutic agent or cholesterol-lowering agent, and a pharmaceutically acceptable excipient for oral administration has been patented [11]. The COX is produced by members of bacterial genera such as *Brevibacterium*, *Cullulomonas*, *Nocardia*, *Pseudomonas*, *Schizophyllum*, *Streptomyces*, *Mycobacterium*, *Rhodococcus* and *Burkholderia*.

Production of extracellular COX in liquid fermentation conditions by *Monascus* X-1 has been reported [4]. An extra-cellular COX producing *Bacillus licheniformis* was isolated from buffalo milk, domestic waste, water sludge and fermented fish sample by enrichment technique [2]. A recent study on COX provided the evidence that a COX isolated from *Bordetella* sp. oxidizes membrane cholesterol to 4-cholesten-3-one with the reduction of O_2 to H_2O_2, resulted in a decrease of cholesterol content and an increase of reactive oxygen species (ROS) levels, which lead to cell apoptosis by inactivation of the Akt and ERK1/2 pathway as well as activation of Caspase-3 [8]. The administration of a COX preferably by oral route oxidizes both dietary and endogenous cholesterol in the gastrointestinal tract to produce 4-cholesten-3-one and H_2O_2. The conversion of cholesterol into 4-cholesten-3-one will prevent cholesterol absorption or reabsorption into the blood stream and effect a reduction of serum cholesterol to subnormal levels. COX from *Streptomyces* sp. for serum assay has been reported to be superior to those from other micro-organisms, due to lower cost of production and longer shelf-life [12]. Ca-alginate immobilized cells of *Streptomyces* sp. studied for the production of COX [15], suggested that immobilized cells could be used for three consecutive fermentation cycles for COX production in higher quantities as compared with free bacterial cells. The studies involving the use of an animal model of cholesterolemia for cholesterol and body weight reduction by use of microbial COX are sparse. We have previously reported purification and characterization of COX from a *Bacillus* sp. COX-T_3 using Riboflavin-affinity chromatography [7]. The purified bacterial COX was successfully immobilized on to Psyllium husk which is white fibrous edible material derived from the outer mucilage coating of the Psyllium seeds. It contains a high level of soluble dietary fiber besides polysaccharides, glycosides, proteins, choline and vitamin B1and is also the chief ingredient in many commonly used bulk laxatives. For developing Psyllium husk bound COX as a functional food supplement for cholesterol lowering, we hypothesized to test the cholesterol lowering efficacy of the purified cholesterol oxidase of *Bacillus* sp. COX-T_3 in an experimental mouse model. In the present study we have shown that administration of purified cholesterol oxidase of *Bacillus* sp. COX-T_3 can lower serum cholesterol level as well decreases body weight in mice.

Materials and methods

Chemicals

$NaNO_3$, K_2HPO_4, KCl, $MgSO_4.7H_2O$, FeSO4. $7H_2O$ and $(NH_4)_2SO_4$ (S.D. Fine-Chem Ltd., Hyderabad, India) $MgCl_2$, cholic acid, riboflavin, sodium borohydried, sodium taurocholate and cholesterol and yeast extract (Himedia Laboratory, Ltd., Mumbai, India); sucrose, KCl, KNO_3, hydrochloric acid and Bromophenol blue (Merck Ltd., Mumbai, India); sodium dodecyl sulfate (SDS), acrylamide, bis-acrylamide (*N*, *N*'-methylenebisacrylamide), glycerol, glycine, Tris (2-hydroxymethyl-2-methyl-1, 3-propanediol), cholesterol oxidase and horseradish peroxidase (Sigma Chemicals Co., USA) were procured from

various commercial suppliers. All chemicals were of analytical grade and were used as received.

Production and purification of extracellular cholesterol oxidase of Bacillus sp. COX-T_3

The *Bacillus* sp. COX-T_3 originally isolated from soil was grown in cholesterol- enriched (CE) broth at 37± 1°C in under continuous shaking (120 rpm) for 24 h. The bacterial cells were sediment by centrifugation and the cell-free culture broth was assayed for extra-cellular activity of COX. The protein content in the broth, cell-free broth or chromatography eluted fractions were determined by a standard method. A previously reported colorimetric method [14] was used to assay the extra-cellular COX in the cell-free broth or approximately diluted commercial grade COX using cholesterol as a substrate. One unit (U) of cholesterol oxidase was defined as the amount of enzyme capable of converting 1.0 ìmole of cholesterol to 4-cholesten-3-one per minute at pH 7.5± 0.1 and at a temperature of 37± 1°C.

Sepahadex G-25 was derivatized to amino-Sepahadex, and then ligand Riboflavin and amino-Sephadex were linked through a spacer (cyanuric chloride) according to a previously described method [9]. This riboflavin-Sephadex gel matrix was used to affinity purify bacterial COX from concentrated preparation of cell-free culture broth of *Bacillus* sp. COX-T_3 as reported recently [7]. The SDS-PAGE (12%) Laemmli [10] was performed to determine the purity of the enzyme.

***In vitro* cytotoxic assay of the purified COX**

To examine the toxic effect of *Bacillus* sp. COX-T_3, if any, cytotoxic assay was performed by exposing Hep2C mammalian cells cultured *in vitro* using DMEM supplemented with 10 % FCS and varying amounts of purified COX [13]. The purple coloured reduced MTT dye entrapped in the monolayer cells was extracted with 100 µL. After keeping the tissue culture plate for 5 min at room temperature the colour was read at A_{570} and the percent viability of the Hep2C mammalian cells was determined.

Application of purified COX for treatment of hypercholesterolemic mouse model

To examine the effect of purified extra-cellular COX produced by *Bacillus* sp. COX-T_3, an experimental hypercholesterolemic mouse model was established using Balb/c mice. Mice were kept on high lipid diet for 14 week with periodic check of serum cholesterol level using a commercial kit (Siemens Healthcare Diagnostic Ltd., 589 Sayajipura, Ajwa Road, Baroda-India) for serum cholesterol enzymatic assay. Mice in the control and the test regimen were fed on placebo (normal pellet diet, Hindustan Lever Ltd., Mumbai) or lipidic diet with water *ad libitum*. Once the hypercholesterolemic mouse model was established, the administration of purified COX of *Bacillus* sp. COX-T_3 *via* intramuscular (I.M.) route at optimized dose was initiated and continued for a period of 35 days after establishment of cholesterolemic mouse model. The serum cholesterol level in these animals were determined for recording therapeutic efficacy, if any to abrogate experimentally enhanced serum cholesterol level in the mouse model.

The mice (n = 30) were divided into six groups of five animals each. The grouping of mice was made as follows;

Group 1: Control regimen was given regular pellet diet and water *ad libitum*; no COX treatment was given to this group.

Group 2: Custom made high fat diet was given for 14 weeks to this regimen along with water *ad libitum*; after 9 weeks of high fat diet feeding each of the mouse was administered COX 0.1 unit/ dose (100 µl) by I.M. route on alternate days for 5 weeks.

Group 3: Custom made high fat diet was given for 14 weeks given to this regimen along with water *ad libitum*; after 9 weeks of high fat diet feeding each of the mouse was administered COX 0.2 unit/ dose (100 µl) by I.M. route on alternate days for 5 weeks.

Group 4: Custom made high fat diet was given for 14 weeks given to this regimen along with water *ad libitum*; after 9 weeks of high fat diet feeding each of the mouse was administered COX 0.3 unit/ dose (100 µl) by I.M. route on alternate days for 5 weeks.

Group 5: Custom made high fat diet for 14 weeks was given to this regimen along with water *ad libitum*; after 9 weeks of high fat diet feeding each of the mouse was administered COX 0.4

unit/ dose (100 µl) by I.M. route on alternate days for 5 weeks.

Group 6: Custom made high fat diet for 14 weeks along with water *ad libitum*; after 9 weeks of high fat diet feeding each of the mice was administered COX 0.5 unit/ dose (100 µl) by I.M. route on alternate days for 5 weeks.

The body weights of the animals in each of the groups were observed on day zero of start of experiment and every week thereafter.

The blood by puncture of retro-orbital plexus of mice using Hematocrit capillaries was drawn on day specified days. After collection of blood in the Hematocrit capillaries, these capillaries were blocked at one end with Plasticin. Thereafter, the capillaries were centrifuged at 3500 rpm for 3 min at room temperature to separate serum. The collected serum was used for the assay of cholesterol.

Data analysis

All the data pertaining to body weight(s) of mice and serum cholesterol contents assigned to the above groups of mice were analyzed by 2-sided Tukey's Honestly Significant Difference (HSD) test values between weeks and animal groups. The different superscript lowercase letters indicated significant difference at $p < 0.05$ between different weeks; and different subscripts lowercase letters indicated significant difference at $p < 0.05$ between different treatment regimens measured by Tukey's test.

Results and discussion

Microbial cholesterol oxidase is an enzyme of great commercial value, widely employed by laboratories routinely devoted to the determination of cholesterol concentrations in serum, other clinical samples, and food. In addition, the enzyme has potential applications as a biocatalyst which can be used as an insecticide and for the bioconversion of a number of sterols and non-steroidal alcohols. The enzyme has several biological roles, which are implicated in the cholesterol metabolism, the bacterial pathogenesis, and the biosynthesis of macrolide antifungal antibiotics **(Doukyu 2009)**. The *Bacillus* sp. COX-T_3 used in the present study produced 0.214 $U.ml^{-1}$ of COX in the broth after 24 h post inoculation. A strain of *Rhodococcus* sp. isolated from soil expressed COX in both extracellular and membrane bound forms. It was reported that a high amount of COX enzyme produced by *Rhodococcus* is extracellular form and only a low amount of COX is membrane bound or intracellular type [17]. Many micro-organisms such as *Nocardia rhodocorus*, *Arthrobactersimplex*, *Pseudomonas* spp., *Rhodococcus* spp., *Coryneform bacterium*, *Actinomyces lavendulae*, *Streptomyces hygroscopicus* and *Brevibacterium* and a few fungal species have been reported to produce COX. It is interesting to notice that a *Bacillus* sp. SFF34 produced two different COXs, both of which were extracellular type of enzymes [19]. *Bacillus cereus* from soil of agriculture waste using cholesterol-Tween-80 medium was used to isolate COX [6]. Thus it seems that some of the *Bacillus* spp. do produce extracellular COX in the natural environment or in an appropriate growth medium.

Affinity purification of cholesterol oxidase of *Bacillus* sp. COX-T_3

The purification of dialyzed COX on a Riboflavin-Sepharose G-25 affinity column resulted in single peak. The pooled fraction represented an activity of 14.9 $U.mL^{-1}$ with a specific activity of 0.135 $U.mg^{-1}$ of protein). The overall purification steps indicated a final yield of ~6.2 % with 28.4-fold purification of extracellular COX of *Bacillus* sp. COX-T_3. The Riboflavin-affinity column purified COX fraction was further evaluated for its electrophoretic homogeneity on gel electrophoresis. Several COXs purified from various bacterial species have been found to possess *M*r in the range of 52 to 61 kDa. A small COX with 31 kDa of *M*r was reported from *Brevibacterium sterolicum* [21].

SDS-PAGE analysis for purity of enzyme

The analysis of bacterial COX under reducing and denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) revealed that the purified COX possessed a single band of *Mr* 21 kDa as visualized with Coomassie Brilliant Blue R-250 staining (Fig. 1). The characteristics of electrophoretic pattern revealed that *Bacillus* sp. COX-T_3 was a homogeneous protein. Two novel extra-

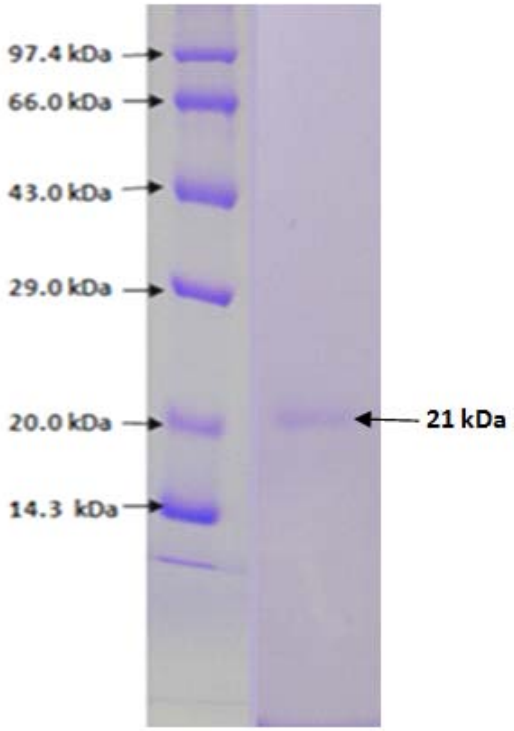

Fig. 1. Sodium deodecyl sulphate-polyacrylamide gel electrophoresis (Stacking T4 % and resolving gel T12 %) of purified cholesterol oxidase of *Bacillus* sp. COX-T_3. Commercially procured protein reference markers (14.3 to 97. 4 kDa) were used to determine the *M*r of the purified enzyme. Left lane: Standard protein markers; right lane: Purified COX of *Bacillus* sp. COX-T_3.

cellular cholesterol oxidases designated CO1 and CO2, from *Bacillus* sp. SFF34, were purified 5.6 and 5.9-fold giving *M*r values of 36 and 37 kDa [19]. The molecular weight of purified COX from *B. cereus* was estimated to be 55 kDa [6].

In vitro cytotoxicity assay of purified COX of *Bacillus* sp. COX-T_3 using a mammalian cell line

When the mammalian cells of Hep2C cell line (a derivative of HeLa cells) grown in DMEM supplemented with FCS (5 %, v/v) were exposed to varying amount of purified COX of *Bacillus* sp. COX-T_3 in *in vitro* had little cytotoxicity when tested at 0.24 to 1.4 $U.ml^{-1}$ concentration in the growth medium at 37± 1°C (Table 1). Interestingly, the addition of the bacterial COX at 1.4 $U.ml^{-1}$ concentrations in the growth medium slightly promoted the cell viability while the bacterial COX at 1.9 $U.ml^{-1}$ in the growth medium exerted a cytotoxic effect on the cell growth as reflected by ~10.6 % decline in the cell viability of mammalian cells recorded in MTT assay. Very limited animal models were reported in the literature for treating experimentally attained hypercholesterolemia created by feeding commercial rations supplemented with 1 % cholesterol or using diets with variations in the lipid and carbohydrate portions, such as different fat sources with or without cholic acid, and partial substitution of sucrose for starch. The rats were hyporesponsive to dietary cholesterol and hence the cholic acid was used in order to induce hyperlipidemia and atherosclerosis in this species. Nevertheless, evidence was presented which did not recommend the utilization of cholic acid in hypocholesterolemic models in rats [18].

Application of purified COX of *Bacillus* sp. COX-T_3 as a therapeutic tool to treat hypercholesterolemia

COX is a useful analytic tool for determining cholesterol in various blood sample; total and esterified serum; from LDL and HDL; on the cell

Table 1. Cytotoxicity of purified cholesterol oxidase of *Bacillus* sp. COX-T_3 towards Hep2C mammalian cells

COX in µL; [U/ml]	0	20 [0.24]	60 [0.72]	100 [1.2]	150 [1.8]
	0.140	0.142	0.142	0.148	0.128
	0.138	0.141	0.133	0.139	0.121
	0.147	0.145	0.149	0.143	0.129
Mean± SD	0.141±0.003	0.142±0.002	0.141±0.002	0.143±0.001	0.126±0.002
Effect on cell viability (%)	-	-	-	‘!12.0	“!10.6

membrane of erythrocytes (and of other cells and cellular compartments) and in gall stones/ human bile. In the present the extra-cellular COX of *Bacillus* sp. COX-T3 was obtained by riboflavin-affinity chromatography and the purified enzyme was intramuscularly administered in an experimentally established mouse model of hypercholesterolemia. The mice were kept on a high lipid diet for an initial 9 weeks followed by another 5 weeks during which the selected group of mice were administered purified bacterial COX (Table 3). The mice of more or less the same age (2.5 weeks old) and average body weight (10.96± 0.79 g) gained 6.7 to 28 % body weight (body weight range 18.61 to 22.32 g/ mouse). After keeping mice assigned to Group 1 to 5 on a high fat diet, the mice showed 14.4, 20.6, 24.4, 19.4 and 17.0 % body weight gain, respectively (mean body weight gain of ~19.0 %) over 9 weeks of intake of high fat feed. The maximum body weight was attained in the mice assigned to Group 5 (22.32± 1.7 g) and fed with high fat diet for a period of 9 weeks in comparison to the control regimen kept on normal pellet diet for the same period of time. These results reflected the successful establishment/ development of a near obese mouse model by intake of a high fat diet for 9 weeks.

Thenceforth, the animals assigned to each of the five treatment groups were administered predefined dose(s) of 0.1, 0.2, 0.3, 0.4 and 0.5 U/ dose of purified enzyme by intramuscular route on alternate days till 5 weeks of scheduled treatment (Table 2). The mice assigned to treatment Group(s) 3, 4 and 5 showed 2.2, 3.5 and 7.2 % decline in their average body weight(s) that indicated a dose/ concentration dependent effect of administration of *Bacillus* sp. COX-T_3 in bringing a reduction in the body weight(s) of these mice (Table 3). The observed results thus provided a potent therapeutic application of purified COX in management of obese subjects as well as bringing a significant reduction in the level(s) of serum cholesterol of these subjects.

The cholesterol level in the high fat diet fed mice significantly increased with age upto 6th week while no significant increase of serum cholesterol content was observed after 6th week in high fat diet fed mice assigned to various groups. For the Group 1 to 5, the high fat diet caused a significant increase of serum cholesterol (Table 3). The reduction of serum cholesterol level by 45.3, 40.9, 22.4, 28.4 and 40.7 % in Group 1 to 5, respectively, was achieved in comparison to the serum cholesterol levels recorded after 9 weeks of intake of high fat diet in mice. Thus the observed data indicated a successful therapeutic application of the purified COX of *Bacillus* sp. COX-T_3 in treating an obese hypercholesterolemic murine model over a reasonable period of enzymatic treatment.

In a previous study, intravenous administration of the COX isolated from *Actinomyces lavendulae* into hypercholesterolemic rabbits at a dose of 0.5-2.0 U/kg body weight decreased distinctly the cholesterol content in blood [20]. The rate of the decrease depended on the dose of the preparation and acute toxicity of the COX preparations to the mice correlated well with the degree of purification of the enzyme and this *Actinomyces lavendulae* COX was suggested to be used as a potential hypocholesterolemic drug. A pharmaceutical composition comprising cholesterol oxi-

Table 2. Effect of normal and high fat diet feed on the body weights (g) of mice

Weeks	Control	G1	G2	G3	G4	G5	Average body weight ±SD
1	$10.60\pm1.34^{a}_{x}$	$11.40\pm2.19^{a}_{x}$	$10.00\pm0.00^{a}_{x}$	$12.00\pm2.74^{a}_{x}$	$10.40\pm0.89^{a}_{x}$	$11.00\pm2.24^{a}_{x}$	10.96±0.79
2	$12.18\pm1.57^{a}_{xy}$	$12.12\pm0.64^{a}_{xy}$	$12.92\pm1.06^{b}_{xy}$	$14.62\pm2.02^{ab}_{y}$	$11.30\pm0.10^{a}_{x}$	$12.74\pm1.70^{ab}_{xy}$	12.74±1.23
3	$12.38\pm1.50^{a}_{x}$	$13.43\pm0.80^{a}_{xy}$	$15.05\pm0.98^{bc}_{xyz}$	$17.02\pm2.19^{b}_{z}$	$14.12\pm0.85^{b}_{xyz}$	$15.56\pm2.04^{b}_{yz}$	15.04±1.38
4	$14.46\pm1.45^{ab}_{xy}$	$13.43\pm0.80^{a}_{x}$	$17.59\pm1.78^{c}_{z}$	$18.73\pm2.23^{bc}_{z}$	$16.53\pm1.02^{c}_{yz}$	$19.04\pm1.48^{c}_{z}$	17.06±2.26
5	$17.44\pm1.75^{bc}_{x}$	$18.61\pm0.60^{b}_{xy}$	$21.05\pm1.11^{d}_{yz}$	$21.63\pm2.27^{cd}_{yz}$	$20.14\pm1.30^{d}_{xyz}$	$22.32\pm1.70^{c}_{z}$	20.75±1.43
6	$19.57\pm2.36^{cd}_{x}$	$21.17\pm1.26^{c}_{xy}$	$24.10\pm0.71^{e}_{yz}$	$25.05\pm2.07^{de}_{z}$	$23.58\pm0.99^{e}_{yz}$	$25.91\pm1.67^{d}_{z}$	23.96±1.79
7	$22.33\pm2.71^{de}_{x}$	$24.58\pm0.85^{d}_{xy}$	$26.81\pm0.81^{ef}_{yz}$	$27.83\pm1.93^{ef}_{z}$	$26.34\pm0.81^{f}_{yz}$	$28.51\pm1.51^{de}_{z}$	26.81±1.51
8	$24.01\pm2.56^{de}_{x}$	$27.16\pm0.93^{e}_{y}$	$29.09\pm0.79^{fg}_{yz}$	$29.79\pm1.05^{fg}_{yz}$	$29.05\pm1.05^{g}_{yz}$	$30.80\pm1.12^{ef}_{z}$	29.17±1.33
9	$26.32\pm2.26^{ef}_{x}$	$30.11\pm0.87^{f}_{y}$	$31.75\pm1.73^{gh}_{y}$	$32.73\pm2.11^{gh}_{y}$	$31.43\pm1.54^{h}_{y}$	$30.80\pm1.12^{ef}_{y}$	31.36±0.98
Body weight change	0.0	↑14.4%	↑20.6%	↑24.4%	↑19.4%	↑17.0%	↑19.2%
10	$28.72\pm2.01^{fg}_{x}$	$31.82\pm0.65^{f}_{y}$	$33.31\pm1.56^{hi}_{y}$	$33.99\pm2.22^{gh}_{y}$	$32.49\pm1.26^{hi}_{y}$	$31.77\pm1.11^{efg}_{xy}$	32.67±0.96
11	$29.48\pm1.87^{fg}_{x}$	$34.12\pm0.45^{g}_{y}$	$34.85\pm1.46^{ij}_{y}$	$34.99\pm1.77^{h}_{y}$	$33.19\pm1.01^{hij}_{y}$	$33.33\pm1.40^{fg}_{y}$	34.09±0.83
12	$31.50\pm2.19^{g}_{x}$	$35.74\pm0.67^{gh}_{y}$	$36.01\pm1.54^{ij}_{y}$	$35.69\pm1.50^{h}_{y}$	$33.93\pm0.79^{ijk}_{xy}$	$34.21\pm1.36^{fg}_{xy}$	35.11±0.96
13	$31.92\pm2.25^{g}_{x}$	$36.23\pm0.80^{gh}_{y}$	$36.44\pm1.50^{j}_{y}$	$36.21\pm1.39^{h}_{y}$	$35.21\pm0.85^{jk}_{y}$	$34.57\pm1.48^{g}_{xy}$	35.73±0.80
14	$32.74\pm1.96^{g}_{x}$	$36.38\pm0.84^{h}_{y}$	$36.77\pm1.01^{j}_{y}$	$36.55\pm1.18^{h}_{y}$	$35.58\pm0.85^{k}_{y}$	$35.11\pm1.30^{g}_{xy}$	36.07±0.70
Body weight change	-	-	-	-	↓2.2%	↓3.5 %	-
Total	22.40±7.96	24.74±9.38	26.12±9.18	26.92±8.63	25.23±8.95	26.12±8.35	25.82±0.85

The observed body weight values were represented as mean ±SD for each column, different superscript lowercase letters indicate significantly different at $p<0.05$ as measured by 2-sided Tukey's HSD between different week for each row, different subscripts lowercase letters indicate significantly different at $p<0.05$, as measured by 2-sided Tukey's HSD between control, Group 1, Group 2, Group 3, Group 4 and Group 5

Table 3. Effect of COX treatment on the cholesterolemic mice

Treatment time (week)	Control	G1	G2	G3	G4	G5
1	$114.13\pm1.00^{a}_{v}$	$180.60\pm1.42^{a}_{y}$	$159.83\pm1.98^{a}_{w}$	$171.10\pm1.61^{a}_{x}$	$168.13\pm1.29^{a}_{x}$	$194.33\pm1.06^{c}_{z}$
2	$122.50\pm2.14^{b}_{w}$	$186.30\pm3.20^{ab}_{y}$	$172.50\pm2.25^{b}_{x}$	$181.33\pm4.34^{a}_{xy}$	$174.40\pm5.31^{a}_{x}$	$200.27\pm3.56^{c}_{z}$
3	$133.73\pm3.03^{c}_{x}$	$184.57\pm2.12^{ab}_{y}$	$183.00\pm3.15^{c}_{y}$	$194.83\pm2.52^{b}_{z}$	$184.60\pm3.47^{b}_{y}$	$201.53\pm3.47^{c}_{z}$
4	$133.97\pm3.76^{c}_{w}$	$189.37\pm1.98^{b}_{x}$	$193.37\pm1.91^{d}_{x}$	$210.27\pm9.77^{cd}_{y}$	$201.00\pm2.07^{c}_{xy}$	$230.90\pm2.02^{d}_{z}$
5	$132.13\pm1.70^{c}_{v}$	$[363.40\pm2.79^{h}_{z}]$	$[265.37\pm2.02^{h}_{w}]$	$[261.57\pm1.85^{f}_{w}]$	$[301.07\pm2.26^{g}_{y}]$	$[276.97\pm2.41^{f}_{x}]$
6	$135.50\pm2.05^{c}_{v}$	$352.47\pm2.53^{g}_{z}$	$254.17\pm1.08^{g}_{w}$	$255.23\pm2.15^{f}_{w}$	$293.53\pm3.07^{g}_{y}$	$273.57\pm2.91^{f}_{x}$
7	$134.33\pm2.10^{c}_{v}$	$336.33\pm2.79^{f}_{z}$	$246.17\pm1.53^{f}_{w}$	$285.03\pm1.70^{g}_{y}$	$264.53\pm4.55^{e}_{x}$	$135.10\pm3.38^{a}_{v}$
8	$135.00\pm1.59^{c}_{v}$	$307.60\pm1.90^{e}_{z}$	$236.43\pm2.73^{e}_{w}$	$239.47\pm1.86^{e}_{w}$	$277.73\pm2.81^{f}_{y}$	$256.07\pm2.55^{e}_{x}$
9	$133.50\pm2.05^{c}_{v}$	$298.60\pm1.20^{d}_{z}$	$230.67\pm2.30^{e}_{x}$	$215.67\pm1.51^{d}_{w}$	$260.40\pm1.91^{e}_{y}$	$232.00\pm1.90^{d}_{x}$
10	$134.10\pm1.54^{c}_{u}$	$[198.63\pm1.05^{c}_{x}]$	$[156.80\pm1.66^{a}_{v}]$	$[202.90\pm1.31^{bc}_{y}]$	$[215.53\pm0.90^{d}_{z}]$	$[164.27\pm0.55^{b}_{w}]$
Body weight change	-	↓45.3%	↓40.9%	↓22.4%	↓28.4%	↓40.7%
Total	130.89±6.98	259.79±75.46	209.83±39.70	221.74±36.06	234.09±49.21	216.50±44.69

The recorded serum cholesterol values were represented as mean ±SD for each column, different superscript lowercase letters indicate significantly different at $p<0.05$, as measured by 2-sided Tukey's HSD between different week for each row, different subscripts lowercase letters indicate significantly different at $p<0.05$ as measured by 2-sided Tukey's HSD between control, Group 1, Group 2, Group 3, Group 4 and Group 5

The observed decline (%) in the serum cholesterol level(s) in the treated groups were compared [with in the group]

dase and co-administration of an HMG-CoA reductase inhibitor, the daily dose of the cholesterol oxidase is approximately 10 mg to 1 g (administered from 1 to 4 times per day), however, this patent did not disclose the equivalent units of enzyme suggested to be used [11]. The observed results clearly indicated that the decline in the serum cholesterol level in the hypercholesterolemic mice was quite gradual; however, there was a significant decline in the body weights of the cholesterol oxidase mice. Approximately 45 and 41 % decline in body weights of the mice assigned to group 1 and 2 was observed that was significant in comparison to the control regimen (Table 3). The study established that the administration of cholesterol oxidase could be successfully used in the weight management and reduction in the serum cholesterol level of the treated subjects.

Conclusion

A purified cholesterol oxidase of *Bacillus* sp. COX-T_3 was successfully employed for the reduction of body weight as well as serum cholesterol in a murine model. The cholesterol oxidase was purified from cell-free culture broth of *Bacillus* sp. COX-T_3 by using a Riboflavin-Sephadex G-25 affinity column. After keeping mice assigned to Group 1 to 5 on a high fat diet, the mice showed approximately 14 to 24 % body weight gain (mean body weight gain of ~19.0 %) over 9 weeks of intake of high-fat feed. The purified COX administered mice at 0.1 to 0.5 U/dose (100 µl) of COX on alternate days for 5 weeks 2.2 to 7.2 % decline in their average body weight(s) along with 22.4, 28.4 to 45.3 % decline in serum cholesterol level. In the MTT assay, the purified bacterial COX showed little cytotoxicity. The study showed that bacterial COX administration in mice may be an effective means of body weight and serum cholesterol reduction. This bacterial COX may be further improved as a therapeutic drug/ molecule to manage/ treat hypercholesterolemic and obese mice by stably binding it with a natural, synthetic or semi-synthetic matrix that could be engineered to provide sustained release of the enzyme *in vivo*.

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Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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