International Journal of Pharmacy and Biological Sciences-IJPBS™ (2019) 9 (3): 277-281
Online ISSN: 2230-7605, Print ISSN: 2321-3272
Research Article | Biological Sciences | Open Access | MCI Approved
UGC Approved Journal

Screening of Actinomycetes for Lipase Inhibitors Production

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Received: 12 Mar 2019 / Accepted: 11 Apr 2019 / Published online: 1 Jul 2019
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Abstract
Obesity has become a serious health problem; it leads to diabetes, cardiovascular & musculoskeletal disorders and some types of cancer. One of the approach for treatment and control of obesity have involved inhibition of dietary lipid digestion by Pancreatic Lipase (PL) inhibitors. Products of natural source provide a vast pool of enzyme inhibitors including pancreatic lipase inhibitors that can be developed antiobesity drug. Actinomycetes are potential sources of enzyme inhibitors, drugs, amino acids, vitamins etc. Present work mainly highlights on the screening of actinomycetes extracts for PL inhibitors. Isolated 110 actinomycetes strains grown in fermentation condition and metabolites are extracted, extract of isolates tested for enzymatic inhibition. 14 extracts have shown positive results for enzyme inhibition and reconfirmed inhibitory activity using enzymatic assay by spectroscopic method. 10 extracts were shown PL inhibitory activity ranging from 10-80%. It concludes actinomycetes are potential source for PL inhibitors, which may lead to valuable novel drugs for obesity treatment.

Keywords
Actinomycetes, Lipase, inhibitors, diabetics, obesity.

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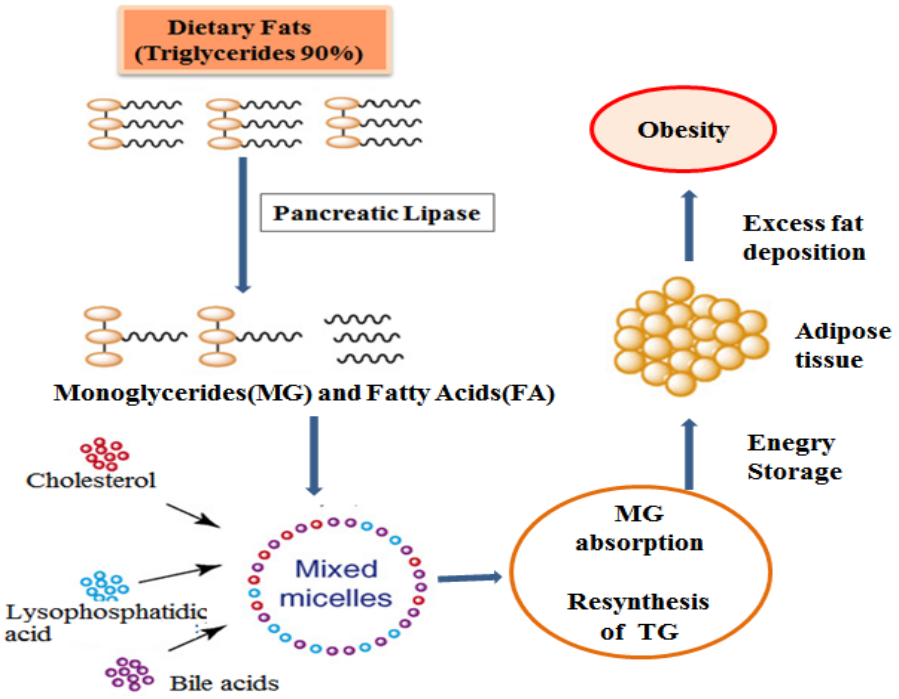
1. INTRODUCTION
Obesity has become a serious health problem in the world. Obesity leads to diabetes, cardiovascular disorders, musculoskeletal disorders and some types of cancer. One of the approaches for treatment and control of obesity have involved inhibition of dietary lipid digestion by Pancreatic Lipase (PL) inhibitors. PL is enzymes that digest fats, including triacylglycerol, phospholipids, and converts to monoglycerides and fatty acids. Physiological role of PL in lipid absorption is depicting in Figure 1.

Figure 1.



Products of microbial and plant source provide a vast pool of enzyme inhibitors including pancreatic lipase inhibitors that can be developed antiobesity drug. Actinomycetes are potential sources of enzyme inhibitors, drugs, amino acids, vitamins etc. Microbial secondary metabolites continue to be a chemically diverse source for the discovery and development of pharmaceutical agents and biochemical probes to study human disease processes (Tamotsu F et al., Cross T.et.al). Actinomycetes have been commercially exploited for the production of pharmaceuticals, neutraceuticals, enzymes, antitumor agents, enzyme inhibitors, and so forth. (Remya M. *et al.*, Rahul B. *et al.,* Hayakawa M. *et al*.) Potential of natural products for the treatment of obesity is still largely unexplored and might be an excellent alternative strategy for the development of safe and effective antiobesity drugs (Rahul B. *et al.*). Actinomycetes can also produce enzyme inhibitors of pancreatic lipase and α amylase to treat obesity, which is a risk factor for hypercholesterolemia, hypertension and diabetes.

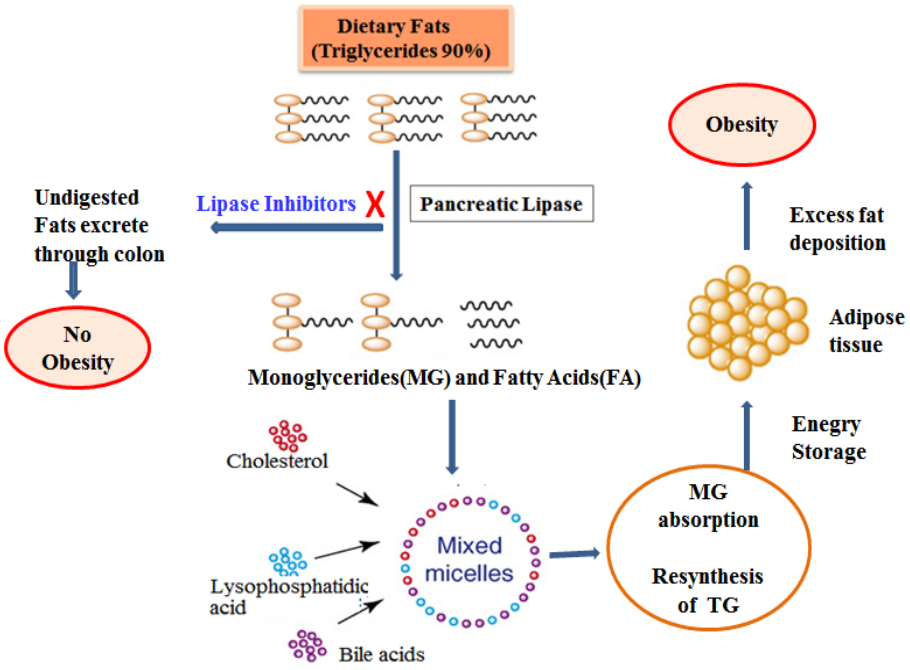
Currently, few FDA approved drugs are available as antiobesity drugs. Table 1. Current antiobesity drugs and their target mechanism. (Rahul B. *et al.*).

Current Antiobesity drugs	Target Mechanism	Company name
Orlistat	Pancreatic Lipase	Roche
Sibutramine	Serotonin and noradrenaline reuptake inhibitor	Abbott laboratories
Rimonabant	CB1 cannabinoid receptor antagonist	Sanofi-Aventis

One of the most important strategies in the treatment of obesity includes development of inhibitors of nutrient digestion and absorption, in an attempt to reduce energy intake through gastrointestinal mechanisms, without altering any central mechanisms .Since dietary lipids represent the major source of unwanted calories, specifically inhibiting triglyceride digestion forms a new approach for the reduction of fat absorption .

PL inhibition is widely studied for the determination of the potential efficacy of different natural and synthetic chemical products as antiobesity agents. Role of PL shown in figure 2.

Figure 2.



Orlistat is clinically approved drugs for obesity treatment, has been shown to act through inhibition of PL. (Heck A *et al*.) Orlistat is one of the best-selling drugs worldwide but it have certain side effects includes unpleasant gastrointestinal side effects, like oily stools, oily spotting and flatulence among others. The success of Orlistat has prompted research for the identification of newer PL inhibitors that lack some of these side effects and more potential effective. Further, there is a need for the isolation of new and novel metabolites from the available natural sources, thus leading to the development of powerful inhibitors. (Nitin A. *et al*.)

Hence present work focusing on the screening of actinomycetes for production of PL inhibitors, which may lead to novel PL inhibitors as anti-obesity drug.

MATERIAL AND METHODS

1. Materials: Porcine Pancreatic Lipase, sodium deoxycholate, sodium phosphate monobasic, isopropanol and p-nitrophenyl palmitate, were purchased from Sigma Aldrich. Media components and reagents purchased from Himedia.

2. Fermentation and extraction of metabolites: Actinomycetes isolated in previous study were stored in 2 to 8°C used for fermentation and metabolites preparation. Culture scraped from slant with 5 ml 2 % peptone solution, 100 µl suspension inoculated in 25 ml medium containing soyabean meal 1.5% (w/v), 2 % Dextrose, 1 % Glycerol in 250 ml flaks, and incubated at 30°C for 48 hrs at 200 rpm on rotary shaker. Production medium inoculated using 10 % grown culture in 25 ml medium in 250 ml flaks for 196 hrs (8 days) at 28°C, triplicate flaks included. The production medium consist of soyabean meal 2.5% (w/v), 0.5 % Yeast Extract, 2 % Dextrose, 1 % Glycerol. After 8 days of fermentation, the metabolites extracted with equal volume of Isopropyl alcohol (IPA) and Ethyl acetate, solvents completely concentrated by rotavapour to get crude material. The collected crude materials stored at 2 to 8°C and used to test Lipase inhibitory activity. The crude extract was dissolved in DMSO to give a stock solution for enzymatic assay.

3. Primary screening: Screening of actinomycetes extract for PL inhibitors was carried out by method developed during this research work based on appearance yellow color of *p*-nitro phenol. This is the screening techniques very quick and don't have lengthy procedure, it required few chemicals. In control reaction tube representing 1 ml enzyme (20 % w/v) and 1ml acetonitrile, test added 100 µL acetonitrile (contains 10 mg extract) in 1 ml enzyme solution, control and test solution incubated at 37°C for 1 hr. 1 ml of *p*-nitrophenol palmitate solution (20 mg in 1 ml acetonitrile) added in preincubated control and test and volume make to 4 ml, reaction tubes incubated at 37°C for 30 minutes. Color changes noted for each extract and compared with the control during incubation and end of the

incubation. A dark-yellow color generate means no enzyme inhibition, fair yellow indicates moderate inhibition & colourless solution indicates strong inhibition of enzyme.

4. Secondary screening: Screening of extracts for PL inhibition by Spectrophotometric assay. The assay was carried out by monitoring the appearance of *p*-nitro phenol or substrate hydrolysis at 400 nm. The principle of the assay is that the substrate, p-nitrophenyl palmitate is hydrolysed by lipase to give a p-nitrophenol (yellow colour). The presence of the inhibitor is indicated by the non-action of the enzyme on the substrate and thereby appearing as a colourless solution.

Enzyme Preparation: Porcine pancreatic lipase was used as a model enzyme; enzyme was dissolved in reaction buffer (10 mg/ml).

Buffer preparation: 100 ml reaction buffer was prepared by adding sodium phosphate monobasic (100 mM), sodium chloride 150 mM and 0.5 ml triton x-100 in distilled water and the final pH of the solution was adjusted to pH 7.4

Assay Protocol: The assay was carried out by monitoring the appearance of *p*-nitro phenol or substrate hydrolysis at 400 nm. The assay mixture (500 µL) contained 50 mM *p*-nitrophenyl butyrate (from stock) and 225 µL reaction buffer (pH 7.4). The reaction was initiated by addition of 100 µL enzyme solution. The fraction containing the inhibitor was dissolved in a minimum quantity of DMSO (10 mg /ml) and added 25 µL in the reaction mixture, DMSO as solvent used for control. Reaction time 3 minutes37°C. Inhibition was expressed as a percentage relative to solvent control. The relative activity was expressed as percentage ratio of enzyme activity in the presence of inhibitors to the enzyme activity in the absence of enzyme inhibitors at the end 3 minutes of the enzyme reaction time.

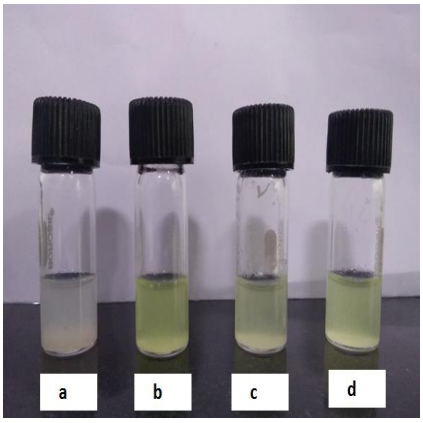
The % inhibition was calculated according to the formula:

$$\text{Inhibition (\%)} = \frac{\Delta A_{540nm}\ \text{(Uninhibited test)} - \Delta A_{540nm}\ \text{(Inhibited test)}}{\Delta A_{540nm}\ \text{(Uninhibited test)}} \times 100$$

RESULTS AND DISCUSSIONS

Primary screening results:

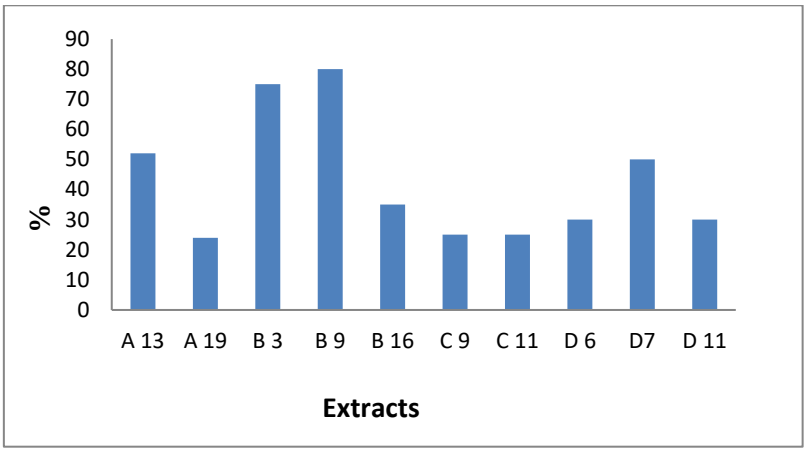
A total of 110 actinomycetes strains were isolated from previous study used for the production of metabolites by fermentation process at shake flaks and metabolites were extracted as described in the materials and methods. Extract of each isolates tested for inhibition of PL in assay method using *p*-nitrophenyl palmitate as substrate. All extracts (110) tested, only 9 extract shown strong enzyme inhibitory activity, 5 extract shown moderate inhibitory activity in simple detection assay method. The inhibitory activity lower to higher measured based colour intensity from yellow to colourless by visual observations. Picture 1: Primary detection of Lipase Inhibitory activity of selected isolates extract. A: Control having enzyme only, b: Enzyme+ substrate, c: extract of B9 + Enzyme + substrate, d: extract of D +Enzyme + substrate.



Secondary screening results:

Out of 110 actinomycetes extract 10 extract shown PL inhibitory activity are again tested for confirmation of Inhibitory activity & determination of percent inhibition of enzyme activity by spectrophotometric method. Extracts shown the inhibitory activity in the range of 10 to 80 % and it shown in the graph no.1.

Graph no.1: Representation of % inhibition of Lipase activity of extracts by spectroscopic method.



CONCLUSIONS

From on-going research, it is confirmed that the actinomycetes produces potential lipase inhibitors, which can be explored as novel antiobesity drug.

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