Effect of Sea Weed Extract on the Production of Lipase using Wild and Mutant Strains of *Bacillus Subtilis* BDG-8

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***Abstract*— Lipases (triacylglycerol acylhydrolases, E.C 3.1.1.3) are currently attracting an enormous attention when compared to other industrial important enzymes. The mechanism of microbial lipase production is generally dependent on cellular regulatory mechanisms elicited by the components of the substrates. Apart from these, different techniques have been used to enhance the production of lipases through the optimization studies involving substrate composition, fermentation parameters and culture conditions. In the present study potential lipase producing bacterial strain *Bacillus subtilis* BDG-8 was isolated from oil spilled soil sample. The selected wild strain *Bacillus subtilis* BDG-8 was subjected to UV irradiation for varying time duration and it was mutated and designated as *Bacillus subtilis* MBDG. In the present study, effect of different seaweeds (*Ulva* sp, *Caulerpa* sp, *Gracilaria* sp and *Sargassum* sp) extract on lipase production by wild and mutant bacterial strains were studied with the production medium supplemented with 1% (v/v) of seaweed extracts. The maximum lipase production of 66.24±0.02UmL^{-1} and 73.22±0.02UmL^{-1} was obtained by the wild and mutant bacterias *Bacillus subtilis* BDG-8 and *Bacillus subtilis* MBDG respectively when grown in crude extract of *Caulerpa* sp supplemented medium. Seaweed concentrates are known to cause many beneficial effects as they contain growth promoting hormones (IAA and IBA, Cytokinins) trace elements (Fe, Cu, Zn, Co, Mo, Mn, and Ni), vitamins and amino acids.**

Keywords— Bacillus subtilis, Ulva sp, Caulerpa sp, Gracilaria sp and Sargassum sp.

I. INTRODUCTION

Among the industrially important enzymes, lipases constitute an interesting class of enzymes with wide biotechnological applications. Lipases (triacylglycerol acylhydrolases, E.C 3.1.1.3) are currently attracting an enormous attention when compared to other industrial important enzymes. This enzyme belongs to the major class of hydrolases having the ability to hydrolyze triacylglycerol to glycerol and free fatty acids over oil- water interface. Potential microorganisms which produce lipases are found in different habitats, including wastes of vegetable oils, dairy product industries, soils contaminated with oils, seeds, and deteriorated food (Sharma et al 2001). The mechanism of microbial lipase production is generally dependent on cellular regulatory mechanisms elicited by the components of the substrates. Apart from these, different techniques have been used to enhance the production of lipases through the optimization studies involving substrate composition, fermentation parameters and culture conditions (Salihu et al 2012). Generally the strategies for improving fermentation process, initial development of an effective medium for lipase production involves selection of carbon source, nitrogen source, inorganic salts, trace elements and growth factors. Even without these, some agro-residues can give satisfactory results but it is common for the appropriate nutrients and optimal concentrations to be established after the initial formulation of the medium (Ooijkaas et al 2000). The reduction in the substrate cost would be a suitable strategy to increase the productivity of any bioprocess techniques (Dominguez et al 2003). Seaweed concentrates are known to cause many beneficial effects on plants as they contain growth promoting hormones (IAA, IBA and Cytokinins), trace elements (Fe, Cu, Zn, Co, Mo, Mn, and Ni), vitamins and amino acids (Pise & Sabale 2010). The nature of the substrate is the most important factor affecting fermentative processes. Thus, process optimization may involve the screening of several alternative substrates (Treichel et al 2010).

The main aim of this study is to isolate and identify the lipase producing bacterial strains from an oil contaminated site and optimizing the production media with different sea weed extracts.

II. EXPERIMENTAL

A. Isolation and Screening of Lipase Producing Bacterial Strains

The potent lipase producing bacterial strains were isolated from soil sample collected from various oil spilled sites. The isolation of bacterial strains were carried out by serially diluting the samples and plating on a tributryrin agar base medium using pour plate method. The plates were incubated at 37°C for 24 hrs and observed for zone formation. A clear zone around the colonies indicates the production of lipase (Cardenas et al 2001). The lipase positive strains were further purified, grown in nutrient broth at 37°C for 24 hrs and screened for the ability to produce lipase with olive oil. Bacterial isolate BDG 8 showed highest lipase activity was identified and further studies were carried out. Morphological, physiological and biochemical characteristics of the potent lipolytic strain BDG 8 was determined by the

method described in "Bergey's Manual of Determinative Bacteriology" (Holt et al 1998). Strain improvement for the selected parent strain was done by mutation. The selected highest lipase produced wild strain BDG-8 was subjected to UV irradiation at varying time intervals 0, 3, 6, 9, 12, 15 and 18 min. The suffix MBDG was designated for the selected mutant strain.

Fermentation was carried out in shake flasks using a complex medium as follows: 20 g l^{-1} olive oil (emulsified in 2% gum acacia), 10 g l^{-1} egg yolk, 4 g l^{-1} ammonium chloride, 0.25 g l^{-1} magnesium sulphate heptahydrate, 0.5 g l^{-1} dipotassium phosphate and 5 g l^{-1} calcium carbonate. The fermentation with an initial pH of 7 was then inoculated with 5 ml of cells suspension and incubated at 37ºC in shaker for 48 hrs.

B. Effect of Different Seaweed Extract on Lipase Production

Different seaweed (macroalgae) such as *Ulva* sp, *Caulerpa* sp, *Gracilaria* sp and *Sargassum* sp were freshly collected from Tamilnadu costal and thoroughly washed in tap water followed by distilled water. Then 1kg of each seaweed was chopped into small pieces and grinded with 400mL distilled water finally made into 1L. The crude extracts were filtered with Whatman No.1 filter paper and stored in the brown bottle at room temperature for further uses. Effect of individual seaweed extract on lipase production studies were carried out in 250 mL Erlenmeyer flask using 100 mL of the production medium contained 1% (v/v) of seaweed extract as substrate. Then the flasks were incubated at 35°C for 48 h at 120 rpm using 1% inoculum. The samples were withdrawn at 12 h intervals, and the biomass was separated by centrifugation at 14,000×g at 4°C for 15 min. The cell free supernatant was used as the crude lipase for determination of lipase activity. The best seaweed was used as substrate for further media optimization.

C. Lipase Assay

Extracellular lipase activity was assayed spectrophotometrically by Krieger et al (1999) method. Samples of the culture medium were withdrawn and centrifuged at 10,000 X g for 15 min. The supernatant was used to estimate the extracellular lipase activity by using *p*-nitrophenyl palmitate (p-NPP) as substrate. The substrate solution was prepared by adding 30mg of p-NPP to 10mL of isopropanol with stirring to dissolve the substrate. This was added to 90mL of 50mM Tris–HCl buffer (pH 8). The mixture of 2.4mL of substrate solution and 0.1mL of appropriate diluted enzyme solution was incubated at 37±1 ºC for 10 min and the reaction was terminated by the addition of 2mL of 0.2 M Na_2CO_3 solution. Released p-nitrophenol (p-NP) was immediately determined by measuring the absorbance at 410 nm in a UV- Visible spectrophotometer. The unknown concentration of 4-nitrophenol released was determined from a standard graph of 4-nitrophenol. All the experiments were repeated three times and graphically represented as mean±SD.

III.RESULTS AND DISCUSSION

Totally eight bacterial colonies were isolated among the five soil samples by serial dilution and plating techniques. Then these isolates were designated as BDG1 – BDG8. After that all the eight isolates were individually subjected to production medium supplemented with 1% (v/v) olive oil and the isolate BDG 8 showed maximum lipase production of 21.23UmL^{-1} was selected. Morphologically BDG 8 showed an irregular, rough flat, opaque colony with an entire margin on nutrient agar plates. It was a gram positive sporulating rod occurring singly or in pairs. It could hydrolyse starch, gelatin, tributyrine, casein, glycerol and could also produce acid in glucose, fructose and sucrose broth. Strain BDG 8 showed positive test for nitrate reduction, citrate utilization and Voges Proskauer test. However it showed negative results for oxidase activity, urea hydrolysis, methyl red test and hydrogen sulphide gas production. Based on the results from morphological, physiological and biochemical characteristics lipolytic strain BDG 8 was identified as *Bacillus subtilis* and it is tabulated in Table:1. The selected wild strain *B. subtilis* BDG-8 was subjected to UV irradiation for varying time duration. A total of 7 isolates of *B. subtilis* BDG-8 were selected and screened for maximum lipase production. The isolate 7 BDG-8 showed maximum lipase production. It was selected as a mutant bacterial strain and designated as *B. subtilis* MBDG and used for further studies.

In the present study, effect of different seaweeds (*Ulva* sp, *Caulerpa* sp, *Gracilaria* sp and *Sargassum* sp) extract on lipase production by wild and mutant bacterial strains were studied with the production medium supplemented with 1% (v/v) of seaweed extracts. The lipase production of 40-60% was observed in all the seaweeds extracts supplemented medium cultures. But the maximum lipase production of 66.24±0.02UmL^{-1}, and 73.22±0.02UmL^{-1}was obtained by wild and mutant bacterias *B.subtilis* BDG-8 when grown in crude extract of *Caulerpa* sp supplemented mediumand the results are tabulated in Figure 1. The biomass concentration of 20.14 g/l and 24.21 g/l was noticed in the bacterias *B.subtilis* BDG-8 and *B. subtilis* MBDG when grown in cured extract of *Caulerpa* sp supplemented medium and the results are given in Figure 2.

Table 1 Morphological and Biochemical characteristics of the isolate

Characters	BDG8
Colony shape	Round
Colony Elevation	Raised
Colony margin	Entire
Cell shape	Rod
Endospore	Positive
Motility	Motile
Grams Staining	Positive
Catalase test	Positive
Oxidase Test	Positive
Methyl red test	Negative
Voges-Proskauer test	Positive
Urease test	Negative
Citrate test	Negative
Triple sugar iron (TSI)	Positive
Indole test	Negative
Dextrose fermentation test	Positive
Isolate identified as	*B. subtilis BDG-8*

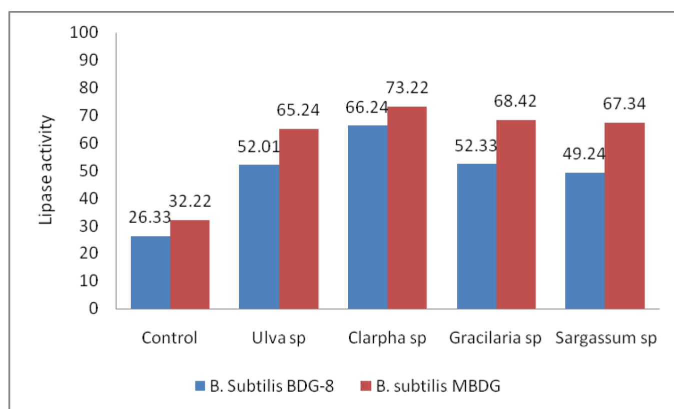

Fig. 1 Effect of crude extract of different seaweeds on lipase production by selected wild and mutant bacterial strain.

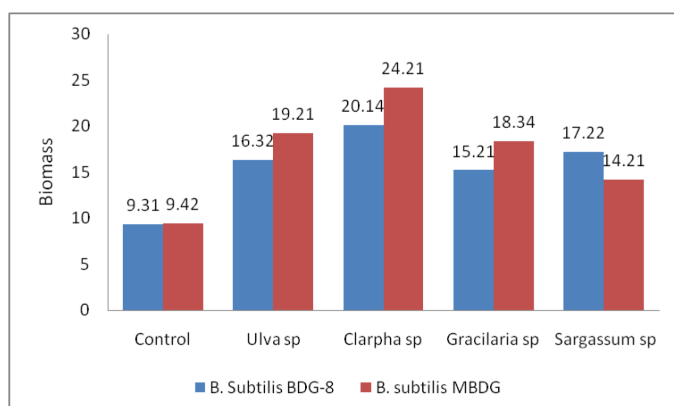

Fig. 2 Effect of crude extract of different seaweeds on the growth of selected wild and mutant bacterial strain

IV.CONCLUSION

Seaweed concentrates are known to cause many beneficial effects on plants as they contain growth promoting hormones (IAA and IBA, Cytokinins) trace elements (Fe, Cu, Zn, Co, Mo, Mn, and Ni), vitamins and amino acids (Pise & Sabale 2010). There was a significant difference in lipase production between wild and mutant strains. The mutant strain produced two fold high lipase than wild strain. The present investigation indicates that the use of seaweed extracts as substrates for bacterial growth and lipase production favours, undoubtedly, the reduction of production costs associated to substrates. Bioconversion of seaweed for lipase production as well as other value added products would hold a prominent position in future biotechnologies, mainly because of its eco friendliness and flexibility to both developing and developed countries.

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