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EVALUATION OF NOVEL IMMOBILIZED LIPASE FROM *Staphylococcus argenteus* MG2 AND ITS APPLICATION AS DETERGENT ADDITIVE

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ABSTRACT

The immobilization of lipase producing alkali tolerant *Staphylococcus argenteus* MG2 bacterial cell and its lipase enzyme was found to have potential applications in industrial field. The immobilization of bacterial cells was done by using sodium alginate. These cells were used for lipase production. The specific activity of lipase enzyme produced by free cells in fermentation was 18.18µM/min/mg. A quite similar activity of lipase (19.55µM/min/mg) was obtained when bacterial cells were immobilized in 3% sodium alginate beads. It was concluded from the study that immobilized cells are effective as free living bacteria for the lipase production. The immobilized crude enzyme solution with diatomite degraded 0.48mg/L (48%) of oil while immobilized cells degraded 0.44 mg/L (44%) of oil which indicates that both immobilized enzymes and immobilized cells were degrading oil efficiently and can be reused easily. It was observed during the study that lipase produced from *Staphylococcus argenteus* MG2 was found to be effective in the formulations of laundary detergent. During the compatibility of lipase enzyme among various commercial detergent tested, intensified activity of lipase was detected with Rin (106.25%) followed by Nirma (103.12%). The stability of lipase was observed towards anionic as well as non ionic surfactants. 50U of lipase in 0.5% Rin improved the removal of oil from oiled cotton fabric. In summary, this novel and potent lipase producing strain isolated from oil contaminated soil, Staphylococcus argenteus MG2, holds immense potential for applications in the detergent industry.

Keywords: Lipase, *Staphylococcus argenteus* MG2, Immobilization, Detergent, Oil removal

INTRODUCTION

Lipase comes from microorganisms and is insoluble in solvent. Immobilized lipase can be recycled and used for continuous production which is stable in solvent also. **(Qiao *et al.*, 1998)**. Cross linking, covalent, entrapping and adsorption methods are used for immobilzation of lipase. Adsorption immobilized process is one of the simplest and economic method **(Chun *et al.*, 2008)**. Diatomite and quartz sand carriers are frequently used in adsorption method **(Chen *et al.*, 2006)**. **Li *et al.*, 2010** used diatomite for immobilization of crude enzyme solution and sodium alginate was used for the immobilization of bacterial cells.

The immobilized biocatalysts (cells) are widely used for the production of extracellular enzymes. The immobilized lipase producing cells could be used for continuous production of lipase at industrial scale. Both immobilized and native lipases are commercially available. Now a days the immobilized cells as biocatalyst have been used in the environmental pollutions **(Rahman *et al.*, 2006)**. The movement of immobilized molecule in space has been restricted **(Zhang *et al.*, 2004)**.

Generally the immobilization supports are divided into organic and inorganic groups. Celluloses, chitosan, dextran, and agarose are examples of organic carriers while zeolite, clay, anthracite, porous glass, activated charcoal, and ceramics are inorganic carriers **(Lu *et al.*, 2009)**. Organic carriers can be divided into natural and synthetic polymers. Alginate, agar, agarose, carrageenan, chitin and chitosan are examples of some natural carriers which can be used as support. Several synthetic polymers are also used for immobilization such as polyurethane, acrylamide, polyvinyl and resin **(Hartman *et al.*, 2005)**. Immobilized cells of *Staphylococcus epidermidis* in agar beads (3%) by entrapment technology enhanced production of lipase as compared to free cells. Immobilized cells with high lipolytic activity and stability may provide commercial advantages over conventional methods of lipase production **(Joseph *et al.*, 2006)**.

Lipases find a major use as additives in industrial laundry and household detergents due to their ability to hydrolyze fats. **Gerritse *et al.*, (1998)** reported an alkaline lipase (*Pseudomonas alcaligenes* M-1) which was efficient to remove fatty stains under conditions of a machine wash. To improve detergency, modern types of detergents usually contain one or more enzymes such as protease, amylase, cellulose and lipase **(Ito *et al.*, 1998)**. *Pseudomonas alcaligenes* (Lipomax®) lipases have been manufactured by Genencor international USA, as detergent additive **(Reetz and Jaeger 1998)**. The usage of enzymes in washing powders still remains the single biggest market for industrial enzymes **(Hasan *et al.*, 2006)**. The latest trend in detergent industry is towards lower wash temperatures which not only save energy, but also help to maintain the texture and quality of fabrics **(Weerasooriya *et al.*, 2012)**. Most of the industrially important lipases are obtained from bacteria (Tab 1).

Table 1 Some commercially avaialable bacterial lipases **(Jaeger and Reetz, 1998)**

Source	Application	Producing Company
Burkholderia cepacia	Organic synthesis	Amano, Fluka, Boehringer, Mannheim
Pseudomonas alcaligenes	Detergent additive	Genencor
Pseudomonas mendocina	Detergent additive	Genencor
Chromobacterium viscosum	Organic synthesis	Asahi, Biocatalyst

A newly isolated strain of *Staphylococcus aureus* (SAL3) has been found to produce a thermoactive, alkaline, and detergent-stable lipase with the capability to hydrolyze its substrate in the presence of various oxidizing agents, surfactants, and commercial detergents. This lipase enzyme, as reported by **Horchani *et al.*,** in **2009**, has potential applications as a detergent additive. Similarly, another *Staphylococcus* species (SL1), isolated by **Cherif *et al.***, in **2011**, demonstrated suitability for detergent formulation.

In a separate study by **Chauhan *et al.*,** in **2013**, lipase isolated from *Staphylococcus arlettae* JPBW-1 was identified as an ideal source for use in the detergent industry formulations. These findings highlight the diversity of lipolytic enzymes and their applicability in various industrial settings. Lipases play a crucial role in processing fats and oils, detergent formulations, degreasing of skin and hides, environmentally safe formulations, food processing, synthesis of fine chemicals and pharmaceuticals, paper manufacturing, as well as the production of cosmetics and pharmaceuticals, as suggested by **Rubin *et al.***, in **1997** and **Kazlauskas *et al.*,** in **1998.**

The catalytic versatility of lipases, as observed by **Saxena *et al.***, in **1999**, underscores the potential for discovering new lipases with specific selectivity and substrate tolerance. This suggests a continual exploration for lipases that meet specific industrial requirements and environmental safety considerations.

Studies on the cost effective production of industrially important lipase enzyme have become the need of today. Looking to the importance of lipase enzyme, this research work was designed with the aims of immobilization of lipase producing bacterial cell and isolated lipase and assay of its use as degrading oil and as a detergent additive.

MATERIAL AND METHODS

Screening of lipase producing bacteria

The potential lipase producing bacterium *Staphylococcus argeteus* MG2 was isolated from oil contaminated soil of oil packing industry of Indore region. This bacterium was able to grow at pH-7 as well as pH-9 Tributyrin agar medium plates which shows its alkali tolerant nature and was used for further study. This isolate was deposited at Institute of Microbial Technology (IMTECH), Chandigarh, India under the accession number MTCC 12820 and its 16S rDNA nucleotide sequence has been deposited in the Gene Bank database (NCBI, US), under the accession number KY082046 **(Golani *et al.* 2019)**. Lipase activity was measured titrimetrically.

Immobilization of bacterial cell and lipase enzymes

Immobilization of bacterial cells by sodium alginate

Sodium alginate was used for immobilization of bacterial cell. 1% inoculum of *Staphylococcus argenteus* MG2 was inoculated in 400 ml of production medium and incubated at 37ºC in orbital shaking incubator at 130 rpm for 48 hours. The culture broth was centrifuged at 10,000g for 30 minutes at 4ºC to get the cell mass. 2.8 gram of mass was mixed with 2.8 ml of saline and with 4% sodium alginate (100 ml), then dropped into $CaCl_2$ solution (0.5 M). The sodium alginate immobilized beads (2 - 2.5 mm) were washed with 0.2 M phosphate buffer saline twice, and then suspended in the same buffer and preserved in refrigerator for reuse. The supernatant of the culture was assayed for the lipase enzyme activity **(Li *et al.*, 2010)**.

Immobilization of crude lipase enzyme solution with diatomite

Immobilization can enhance enzyme stability and activity. The immobilization of lipase enzyme was done by using diatomite. 08 g of dried diatomite was added to 100ml of supernatant of lipase produced in production medium **(Golani *et al.*, 2016)**. It was agitated for 3 hours at 30-35ºC and then centrifuged to get precipitate which was washed by acetone for 3-5 times to make particle dispersed and stored at 4ºC **(Li *et al.*, 2010)**.

Production of lipase via immobilized cells

The 100ml of production media was inoculated with 3% immobilized beads of *Staphylococcus argenteus* MG2 cells, incubated at 37ºC in orbital shaking incubator at 130 rpm for 48 hours. This medium was centrifuged at 10,000g for 30 minutes at 4ºC. The supernatant of the medium containing lipase was assayed for the lipase enzyme activity.

Degradation of oil by immobilized cells and immobilized enzymes

1 gm of olive oil was added in 100ml of 0.1 M phosphate buffer (pH-7.2). This sterile medium was inoculated with 3% of immobilized beads of *Staphylococcus argenteus* MG2 cells, incubated at 37ºC in orbital shaking incubator at 130 rpm for 48 hours. This medium was centrifuged at 10,000g for 30 minutes at 4ºC. The similar experiment was performed for immobilized enzyme also. The supernatant collected was used to check the degradation of oil by using partition gravimetric method.

Oil quantification

The quantity of oil present in supernatant was determined of by using partition gravimetric method of **Kirschman and Pomeroy (1949)**.

APPLICATION OF ALKALINE LIPASE AS DETERGENT ADDITIVE

Stability of the lipases in different commercial detergents

The compatibility of the lipase enzymes as partially purified ammonium sulphate precipitated fraction was studied with commercial detergents **(Sharma *et al.*, 2002a)**. The commercial detergents used for the study included Nirma, Rin, Wheel, Tide, Ariel, and Surf excel that are of common use in India. The lipase enzyme under study was incubated with each of the mentioned detergent preparations (10 mg/ml, w/v) in the proportion of 1:1(v/v) for 30 minutes at 37ºC temperature for the specific activity of lipase enzyme. The native enzyme present in the detergent was inactivated by heating at 100ºC for 10 minutes. Thereafter, the resultant activity of lipase was determined by titrimetric assay method and compared with the control (without any detergent) which was incubated under same condition. The relative activity (%) of control was taken as 100%.

Stability of the lipases in the presence of oxidizing agents

Stabiity of lipase (50U/ml) in the presence of oxidizing agents was analyzed in Tris-Hcl buffer (0.1M, pH 8.0) consisting 0.5-2.0% of hydrogen peroxide, sodium perborate, and sodium hypochlorite, incubated for 1 hour at 37ºC, and the resultant activity of lipase was determined by titrimetric assay method and compared with the control (without oxidizing agent). The relative activity (%) of control was taken as 100% **(Rathi *et al.*, 2001)**.

Wash performance analysis

The fabrics used for the analysis of the wash potential study of the lipases included cotton fabrics. The fabrics were cut into 5 x 5 cm^2 pieces and each piece was stained by spotting with 200 µl of oil-dye mixture which were dried for 15 minutes at room temperature. The oil-dye mixture was prepared by solubilizing 200 mg of the oil-soluble dye, Sudan III in 10 ml each of olive oil and mineral oil on a vortex mixture **(Neeru *et al.*, 2001)**. The dried stained fabric pieces were put separately in 250 ml beakers each containing a specific washing solution i.e. B, BD, BL, BDL and BDIL (Tab 2) prepared using the detergent in which the lipase was found to be the most stable.

Table – 2 Formulation of the washing solutions

Constituents	Volume (ml)				
	B	BD	BL	BDL	BDIL
Tris-Hcl buffer (0.1M, pH 8.0)	40	40	40	40	40
Detergent solution (0.5%)	-	50	-	50	50
Lipase (50U/ml)	-	-	5	5	-
Immobilized Lipase (gm %)	-	-	-	-	0.5
Distilled water	60	10	55	5	10

B: Buffer, BD: Buffer + Detergent, BL: Buffer + Lipase, BDL: Buffer + Detergent + Lipase, BDIL: Buffer + Detergent + Immobilized lipase

The beakers were incubated for 20 minutes and 40 minutes at room temperature to remove the stains from the fabrics. After every 20 minutes, fabric pieces subjected to each specific set of washing conditions were taken out, washed gently in flowing tap water, dried at room temperature and the extent of oil-dye stain removal was assessed by visualization and recorded as no cleaning (-), weak cleaning (+), moderate cleaning (++), good cleaning (+++) and excellent cleaning (++++) potential.

RESULTS AND DISCUSSION

Immobilization of bacterial cell and lipase enzymes

Immobilization of bacterial cells by sodium alginate

Alginates (extracted from brown algae) are mostly used for immobilization of cells because it is easily available, nontoxic to humans, and cheap also. From a physiological standpoint, alginate offers a significant benefit as immobilized cells undergo minimal alterations in physicochemical conditions throughout the immobilization process, and the gel maintains transparency and permeability. **(Buque *et al.*, 2002)**. Entrapment method was used for the immobilization of cells which has been widely investigated **(Jack *et al.*, 1977)**. The support matrixes of alginates have porous structure which allows easy diffusion of pollutants and various metabolic products **(Verma *et al.*, 2006)**. The immobilized bacterial cells within sodium alginate beads were small, spherical, and white, facilitating their easy separation from the reactant. (Figure 1).

Immobilization of crude lipase enzyme solution with diatomite

Diatomite – immobilized crude enzyme was like white powder which was not soluble in organic solvent and water (Figure 2).

Production of lipase via immobilized cells

The goal of bacterial cell immobilization is to enable prolonged utilization as a biocatalyst. In fermentation, free cells (approximately 4.8×10^9 cells/ml) produced lipase with a specific activity of 18.18 µM/min/mg. A comparable lipase activity (19.55 µM/min/mg) was attained when cells were immobilized within 3% alginate beads, with a cell biomass of 2.8×10^9 cfu g^{-1}.

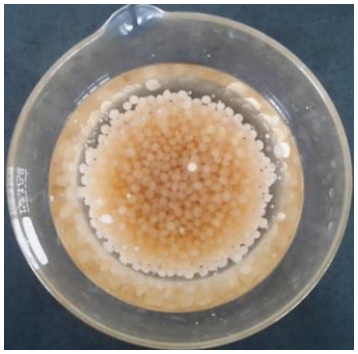


Figure 1 Immobilization of Bacterial cell

Figure 2 Immobilization of Lipase

a **b**

Figure 3 Degradation of oil by (a) immobilized cells and (b) immobilized enzyme

In our studies, production of Lipase via immobilized cells at different intervals of storage time period in months was checked (Tab 3). Initially the lipase activity (19.55 µM/min/mg) was high which gradually decreased after months but immobilized living whole cells have a great potential for lipase production and very easy to store it in refrigerator for several months. Even after 12 months immobilized cells were in live condition and were able to produce lipase enzyme and lipase activity was found to be 1.89µM/min/mg. Therefore, it can be inferred that immobilized cells, in contrast to free-living bacteria, offer enhanced effectiveness, an extended shelf life, and a reduced production cost for lipase. This advantage arises from the elimination of the need to repeatedly prepare cultures.

Table 3 Production of Lipase via immobilized cells at different intervals of storage

Months	Lipase activity (µM/min/mg)
0	19.55
2	16.23
4	12.65
6	9.12
8	5.48
10	3.33
12	1.89

Degradation of oil by immobilized cells and immobilized enzymes

The immobilized enzyme and bacterial cell, having equal weight (3%) was added individually for the degradation of oil to the 100 ml of phosphate buffer containing 1 gm of olive oil (Figure 3). After completion of optimum time period, the content of oil degraded (mg/L) values by immobilized enzymes and immobilized cells of *Staphylococcus argenteus* MG2 was checked by partition gravimetric method of **Kirschman and Pomeroy (1999)**. The results showed that immobilized crude enzyme solution with diatomite degraded 0.48mg/L (48%) of oil while immobilized cells degraded 0.44 mg/L (44%) of oil which indicates that both immobilized enzymes and immobilized cells were degrading oil efficiently and can be reused easily. Thus, it can be concluded that immobilized enzymes and immobilized cells have crude oil degrading activity.

Application of alkaline lipase as detergent additives

Stability of the lipases in different commercial detergents

In the detergent industry, it is imperative for lipolytic enzymes to exhibit compatibility and stability with the various ingredients commonly employed in detergent formulations **(Kamini *et al*., 2000)**. An ideal detergent enzyme is anticipated to maintain stability when exposed to commercial detergents. To assess compatibility, enzyme solutions were incubated with deactivated detergents at a final concentration of 10 mg/ml, at 37ºC for 30 minutes. Subsequently, the enzymes' compatibility with commercial detergents was evaluated. As per the Indian standard, the detergency percentage in commercial detergent was: Nirma- 38.60%, Rin- 54.01%, Wheel- 52.43%, Tide- 57.98%, Ariel- 72.29%, and Surf excel- 67.54% respectively.

Among the different types of detergents examined, increased lipase activity has been analyzed with Rin (106.25%) followed by Nirma (103.12%). However, the residual activity in presence of other detergents viz. Tide (81.25%), Wheel (75%), Ariel (71.87%), and Surf Excel (71.87%) decreased respectively (Figure 4).

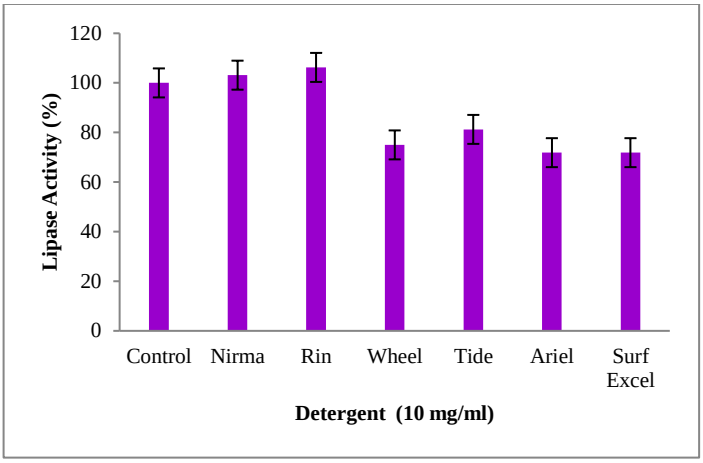


Figure 4 Stability of the lipases in different commercial detergents

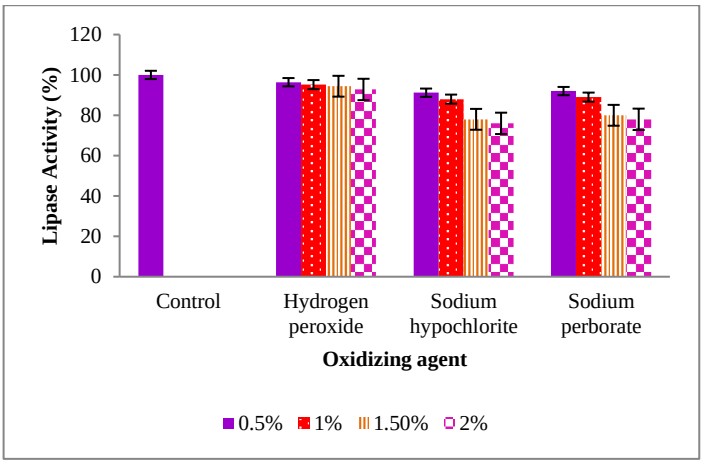


Figure 5 Stability of the lipases with oxidizing agents

Stability of the lipases with oxidizing agents

The stability of lipase in the presence of oxidizing agents, such as hydrogen peroxide, sodium hypochlorite, and sodium perborate, was assessed. Remarkably, the lipase exhibited high stability at a 1.5% concentration for 1 hour at 37ºC, retaining 92.8% of its activity even at a 2.0% concentration of hydrogen peroxide. However, the activity gradually declined with an increase in the concentration of sodium perborate and sodium hypochlorite from 1.0 to 2.0% (Figure 5). Consequently, the higher stability in the presence of oxidizing agents positions this alkali-tolerant lipase as an ideal enzyme for incorporation into any detergent formulation, promising enhanced results.

Wash performance analysis

Among the various detergents tested, Rin (0.5%) exhibited heightened lipase activity and was consequently selected for further studies. To evaluate the applicability of lipase as a wash detergent additive, an experiment was conducted involving cotton fabric pieces stained by spotting an olive oil-dye mixture for 15 minutes at room temperature. The impact of washing time on oil removal is presented in Tab 4.

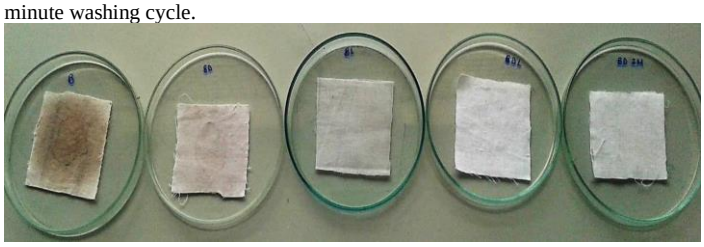
Table 4 Influence of lipase on the removal of olive oil from cotton fabric using Rin detergent (0.5%)

Time (minutes)	B	BD	BL	BDL	BDIL
20	-	+	++	+++	++
40	-	+	++	++++	+++

No cleaning (-), weak cleaning (+), moderate cleaning (++), good cleaning (+++) and excellent cleaning (++++)

It has been observed that in 0.5% Rin detergent solution (Figure 6), cleaning with B-D-L solution was good and moderate with both B-L and B-D-IL solution than B-D solution (weak) in 20 minutes. In conclusion, it has been noted that the

application of B-D-L solution leads to improved oil removal, particularly with a 40 minute washing cycle.



B BD BL BDL BDIL

Figure 6 Influence of lipase on the removal of olive oil from cotton fabric using Rin detergent (0.5%**)**

DISCUSSION

Immobilization of bacterial cell and lipase enzymes

A higher yield of lipase with activity (19.55µM/min/mg) was achieved when cells were immobilized in 3% alginate beads with a cell biomass of 2.8×10^9 cfu g^{-1} than the specific activity of lipase enzyme produced by free cells (Approximately 4.8×10^9 cells/ml) in fermentation was 18.18µM/min/mg. It was concluded from the study that immobilized cells compared with free living bacteria are effective, have longer shelf life and lower cost price for the production of lipase because there is no need to prepare culture repeatedly.

Our findings align with those of **Joseph *et al.*, 2006**, who reported that cells of immobilized *Staphylococcus epidermidis* in agar beads (3%) exhibited an increased lipase production of 11.7U compared to free cells, which produced 8.1U. Notably, *Staphylococcus epidermidis* cells immobilized on polyacrylamide displayed lower production, likely attributed to the diminished viability of the cells. Furthermore, our study revealed that whole cells within a gel matrix-maintained viability for up to a month under specific conditions. The application of immobilized living whole cells holds potential for innovation in a novel fermentation process.

The immobilized crude enzyme solution with diatomite degraded 0.48mg/L (48%) of oil while immobilized cells degraded 0.44 mg/L (44%) of oil which indicates that both immobilized enzymes and immobilized cells were degrading oil efficiently and can be reused easily.

As per **Fathi *et al*., (2021)**, the calculated immobilization efficiency of the lipase enzyme from *Lactobacillus fermentum* using chitosan polymer was remarkably high at 56.21%. This substantial percentage underscores the exceptional capacity of the chitosan polymer to effectively capture proteins, including the lipase enzyme. However, after a 24-hour period, the efficiency of stabilized lipase activity reduced to 44.6%, signifying that this technique is well-suited for the long-term preservation of the lipase enzyme.

The immobilized cells and enzyme both were used for the degradation of oil and concluded that immobilized enzymes and immobilized cells have crude oil degrading activity.

Application of alkaline lipase as detergent additives

The evaluation of enzyme compatibility with commercial detergents revealed superior lipase activity enhancement with Rin (106.25%) and Nirma (103.12%) among the tested detergents. Notably, the lipase demonstrated stability not only towards non-ionic surfactants like Triton X-100 and Tween 80 but also towards the potent anionic surfactant SDS, oxidizing agents, and commercial detergents **(Golani *et al*., 2016)**.

The lipase exhibited remarkable stability against oxidizing agents, retaining 92.8% of its activity at 1.5% hydrogen peroxide concentration for 1 hour at 37ºC. Even at a higher concentration of 2.0%, the enzyme retained significant activity. However, a gradual decrease in activity was observed with increasing concentrations of sodium perborate and sodium hypochlorite from 1.0 to 2.0%. Interestingly, the lipase displayed superior resistance to strong oxidizing agents, particularly hypochlorite, with 88% activity at 1.0% concentration. In comparison, Lipolase (Novozymes, Denmark) exhibited only 43% activity after 1 hour of treatment, as reported by **Rathi *et al*., 2001**.

The study results indicated that the lipase from *Staphylococcus argenteus* MG2 enhances oil removal from oiled cotton fabric when used as an additive to the commercial detergent, specifically 0.5% Rin, under optimal conditions of 50U of lipase in Tris-HCl buffer (0.1 M, pH 8.0). Furthermore, the significant contribution of the lipase to oil removal was observed with prolonged washing times, aligning with findings reported by **Chauhan *et al*., 2013**, in the case of *Staphylococcus* sp. lipase bio detergent study, and by **Saraswat *et al*., 2017**, in the case of *Bacillus subtillis* strain.

CONCLUSION

The study concludes that immobilized cells, in comparison to free-living bacteria, exhibit greater effectiveness, prolonged shelf life, and a lower production cost for lipase. Both immobilized cells and enzymes were employed for oil degradation, revealing heightened crude oil-degrading activity with immobilized enzymes and cells. The immobilized enzyme, derived from *Staphylococcus argenteus* MG2, was identified as suitable for detergent formulations.

The lipase produced by *Staphylococcus argenteus* MG2 emerges as an ideal candidate for inclusion in laundry detergent formulations, showcasing high activity and stability at elevated alkaline pH levels and temperatures. Notably, the lipase proved stable not only against non-ionic surfactants but also against the potent anionic surfactant SDS, oxidizing agents, and commercial detergents. The study's findings indicate that incorporating lipase from *Staphylococcus argenteus* MG2 enhances oil removal from oiled cotton fabric when added to commercial detergent formulations. Consequently, the lipase from *Staphylococcus argenteus* MG2 is deemed an optimal choice for formulating environmentally friendly detergent formulations aimed at triglyceride oil removal from fabrics.

In summary, the isolated *Staphylococcus argenteus* MG2 demonstrates potential as a bacterial source of lipase, with its alkali-tolerant nature making the enzyme suitable for detergent formulations and various industrial applications.

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Conflict of interest: The author declares no conflict of interest.

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