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RESEARCH ARTICLE

OPTIMIZATION OF PROCESS PARAMETERS FOR ENHANCED THERMOSTABLE LIPASE PRODUCTION BY BACILLUS SUBTILIS SHVSC04.

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Abstract

Thermophilic lipases are stable at higher temperatures, which enhance their demand in industrial applications. In the present study, thermostable lipase was produced from bacterial strain *Bacillus subtilis* SHVSC04 (MN565992) isolated from Tuva Timba hot spring, Gujarat, India. Isolate displayed maximum growth in basal medium augmented with 3% sucrose, 3% yeast extract and 2 % salt at pH 7 and 50°C in 48 h. Whereas, isolate produced maximum lipase in tributyrin agar medium with pH 7, enriched with 3% sucrose, 3% yeast extract and incubated at 50°C in 72 h. Tributyrin (2%) was found to be the best substrate for lipase production in submerged conditions. The amount of lipase was increased by 1.5 fold upon optimization of different environmental and nutritional parameters. The enzyme retained 84% activity at 60°C and 70% of activity at 70°C for 1h. The present findings advocate that hot springs in Gujarat are a substantial source of thermostable bacteria producing enzymes of industrial importance.

Introduction:-

Lipases (triacylglycerol acyl hydrolases, E. C. 3.1.1.3) are important group of enzymes, which can catalyze both hydrolysis and ester synthesis. In aqueous environment it hydrolyzes triacylglycerols and release fatty acids and glycerol, while in non-aqueous environment, it catalyze conversion reactions viz., transesterification, esterification, interesterification, aminolysis, alcoholysis and acidolysis. Lipase enzyme used as a catalyst for production of diverse products in different industries including fats and oils processing, detergents and degreasing formulations, food processing, fine chemicals production, paper fabrication and preparation of cosmetics and pharmaceuticals (Tripathi et al., 2014). Extracellular lipases are generaly distributed among animals, plants and bacteria. Amid these, bacterial lipases have gained appriciable industrial importance due to high production, high adaptability, high stability and rapid growth with alleviate of genetic manipulation (Shaini and Jayasree 2016). Hence, lipase producing bacterial strains has been widely studied from diverse habitats such as hot springs, decaying food, compost heaps, oil factories, dairies, soil contaminated with oil and used industrially for an extended time (Wang et al., 1995).

It is been reported that mesophilic enzymes are unstable in many industrial processes which, are performed at harsh conditions i.e. extreme temperature, pH, concentrations of organic solvents and detergents (Si et al., 2018). Thermostable enzymes have achieved precedence over thermolabile enzymes due to their ability to endure higher

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temperatures, confer a longer half-life to the enzyme and decrease the possibility of microbial contamination in industrial processes (Mistry et al., 2016). Thus, it advocates the quest for a unique thermostable enzyme producing microbial isolates in the industrial processes. The organisms that produce these exceptional enzymes are recognized as thermophiles, habituated in hot springs which, survive in temperatures above 50°C.

However, these microbes have been less explored due to complications in isolation and preservation; nevertheless, certain Bacillus species have been reported to produce useful enzymes (Rahman et al., 2007). Rathi et al., 2001 reported lipases from Burkholderia sp., which have been accentuated due to the remarkable thermal stable and solvent tolerant properties. Other additional species- *Aneurinibacillus thermoaerophilus, Geobacillus thermoleovorans, Geobacillus thermodenitrificans, Bacillus thermoleovorans,* are reported as potent isolated for industrial thermostable lipase (Masomian et al., 2013; Abol Fotouh et al., 2016; Christopher et al., 2015; Tripathi et al., 2014).

In pursuit of thermostable lipase, in present article we have described production and characterization of *Bacillus subtilis* SHVSC04 with media optimization for maximum growth and high lipase productivity. The aim of the present study was to isolate an efficient thermophilic lipolytic bacterium and optimize the enzyme production from isolated strain.

Materials and Methods:-

Material

Nutrient agar, tributyrin oil, gum arabic, bile salt and bovine serum albumin (standard protein) were procured from Himedia, India. Olive oil, castor oil, coconut oil, and groundnut oil were purchased from the local market. All chemicals and solvents used in the experiment are of analytical grade. The instruments being utilized include Centrifuge and Incubator of REMI, Microscope of Equitron, Autoclave of Magnus Company and Spectrophotometer of SIMATZU.

Isolation and screening of lipase producing bacteria

The bacterial culture used in the present study was isolated by screening lipase producing bacteria from water samples of Tuva Timba (22°47'58"N and 73°27'37"E), a hot spring of Gujarat, India. For enrichment, the water sample was inoculated in nutrient broth followed by spread plating on nutrient agar and incubating at 50°C till visible growth was achieved. The thermotolerant organisms thus acquired, were then subjected to further screening of potential lipase producer using tributyrin agar assay method (TBA), where isolates were streaked on tributyrin agar media plate, supplemented with 1% (V/V) tributyrin and incubated at 50°C for 24 h to observe zone of hydrolysis.

Identification of isolated bacterium

The most efficient bacterial strain with the ability to produce thermostable lipase was selected for further study. This isolate was then identified using morphological, cultural and biochemical characterization as per Bergey's Manual and further identification was confirmed by 16S rRNA gene sequence analysis. The amplification of the 16s rRNA gene was performed in the BioRad PCR Cycle. The amplified PCR product was subjected to sequencing by automated DNA analyzer. The obtained sequence was analyzed using the SEQMATCH tool of the Ribosomal Database Project (RDP-II). The organism was identified as *Bacillus subtilis* SHVSC04 (MN565992).

Optimization of growth conditions of isolate

Optimization of Nutritional factors

The media was optimized by varying one component at a time, for the maximum growth of *Bacillus subtilis* SHVSC04. To find the best carbon source for promoting growth, the isolate was inoculated in basal media enriched with peptone (1% W/V) along with one of the carbon sources such as glucose, sucrose, maltose, lactose, mannitol, and dextrose. Similarly, to find the best nitrogen source, media was complemented with sucrose (1% W/V) along with one of the five nitrogen sources: peptone, ammonium sulphate, Yeast Extract, urea, ammonium nitrate, and ammonium phosphate (1% w/v). For the optimum quantity of sucrose & yeast extract, a range of concentrations (1 to 7 %) was individually supplemented in the basal media. To examine salt tolerance, the bacterial strain was inoculated in media containing different NaCl concentrations ranging from 1 to 7 %. At the end of 24 hours incubation period, culture was harvested to analyze growth by measuring OD at 600nm.

Optimization of Environmental factors
The bacterial strain was grown in production medium containing 3% sucrose, 3% yeast extract and 2% NaCl to evaluate the effect of various environmental factors on lipase production. To investigate effect of temperature on growth, the culture was incubated at different temperatures including 30, 40, 50, 60 and 70°C for 24 hours. The effect of pH was monitored by using media with pH range of 4 to 9, after 24 hours of incubation growth of isolate was analyzed by measuring OD at 600nm.

Lipase production
Screened isolate *Bacillus subtilis* SHVSC04 (MN565992) was cultivated in lipase producing media for its production. The composition of media for lipase production was as follows (g/L): peptone (10), glucose (10), NaCl (5), yeast extract (5), KH_2PO_4 (1), K_2HPO_4 (0.3), $CaCl_2$ (2), $(NH_4)_2SO_4$ (2), $MgSO_4.7H_2O$ (0.2) and trybutirin oil 2% (v/v), pH -7.4. Inoculated culture was incubated at 50^0 C for 24-48 h and centrifuged to collect crude enzyme. The crude extract was analyzed for lipase activity by the titrimetric assay method.

Lipase Assay:
Lipase was assayed by constant monitoring of the fatty acid released from tributyrin by titration against NaOH. The assay mixture contained: 1% emulsion where, tributyrin oil (10% v/v) was emulsified with 5% w/v gum Arabic and 100mM Tris Cl buffer, pH 7.0.One unit of enzyme is defined as the amount of enzyme required to hydrolyze µmol of fatty acids from triglycerides. Protein content was measured by Lowry's method using BSA as standard (Lowry et al., 1951).

Effect of nutritional and environmental factors on lipase production
To obtain maximum lipase production from *B. subtilis* SHVSC04, different parameters were studied.

Effect of substrate:
Five different substrates were analyzed for maximum lipase production viz. coconut oil, castor oil, olive oil, tributyrin oil and groundnut oil. Studies were also executed to estimate the influence of different tributyrin concentration (1–5 %, (w/v)). Each following factor was examined as per optimized conditions with all experiments were done in triplicate.

Effect of incubation period:
Lipase production was analyzed at 50°C for 6 days. At the end of each 24 hours of incubation period, culture was harvested and centrifuged to estimate lipase production. Out of different incubation periods, the optimum incubation period for maximum enzyme production was considered for further studies.

Effect of different Carbon source:
This isolate was inoculated in production media, supplemented with various carbon sources such as glucose, sucrose, maltose, lactose, mannitol, and dextrose, to find the best carbon source. The study was also performed to estimate the influence of different sucrose concentrations (1– 5 % (w/v)).

Effect of different Nitrogen source:
The effect of five different organic and inorganic nitrogen sources viz. peptone, ammonium sulfate, Yeast Extract, urea, ammonium nitrate, and ammonium phosphate (1% w/v) was studied for lipase production. The investigation was also executed to estimate the influence of different yeast extract concentrations (1– 5 %, (w/v)).

Effect of temperature:
To investigate the effect of temperature on lipase production, the culture was incubated at different temperatures including 30° C, 40° C, 50° C, 60° C and 70° C for 5 days and the lipase activity was estimated after every 24 hours.

Effect of pH:
The effect of pH was monitored by using media with a pH range of 4 to 8 for 5 days; the enzyme activity (U/ml) was measured after every optimization phase, as per the standard enzymes assays.

Results and Discussion:-

Isolation and screening of thermotolerant bacteria for Lipase production

The 7 isolates obtained from Tuva timba, a hot spring of Gujarat (Fig 1a) were screened for lipase production on tributyrin agar plates. Amongst them, 4 isolates were found to be lipase producers and designated as TH2 to TH5. Out of four isolates, with the zone of clearance on the tributyrin agar plates, the isolate TH2 exhibited a superior zone of clearance (n/z) of 3 mm (Fig 1b) along with maximum lipase activity of 12 U/ml, and therefore used for further experiments. It was grown maximally at 50°C and can tolerate temperature till 70°C, which indicates the thermotolerant nature of an isolate. Patel D et al. 2019, reported the cultivation of 48 isolates using various media from Tuva timba, a hot spring of Gujarat, with the potential biotechnological and industrial applications. Ahmed et al., 2010 also isolated thermotolerant alkaline lipase from *Bacillus subtilis* EH 37 from oil-rich soil samples.

Identification of *Bacillus subtilis* SHVSC04

The isolate was preliminarily characterized based on its colony morphology and culture characteristics with dry, flat, irregular, with globate margins and white coloured colonies. Further, microscopic examination through different staining techniques revealed that the TH2 strain is endospore-forming, capsulated, motile Gram-positive rods. The biochemical tests as per Bergey's manual of systematic bacteriology, the isolated bacterial strain was recognized as Bacillus species (Refer to table 1). Cappuccino et al., 1996 supported the identification of such genera. In agreement with Bergey's Manual of systematic bacteriology, 16S rRNA, 1505 bp, sequence evaluation using the SEQMATCH tool of Ribosomal Database Project (RDP-II) confirmed the strain as *Bacillus subtilis* SHVSC04, with the NCBI GenBank accession number of MN565992. The sequence-based phylogenetic tree of 16S rRNA gene sequence is presented in Fig 1c.

Numerous reports deliberate that thermotolerant Bacillus species, which exist in Gujarat coasts, sea, and hot springs are a rich source of lipase. Patel et al. 2019, identified various isolates from Tuva Timba, hot spring of Gujarat, encompassing the genera- *Bacillus*, *Brevibacillus*, *Geobacillus*, *Anoxybacillus*, and *Brevibacterium*. In addition to, Mangrola et al., 2015, also discovered 29 bacterial species belonging to the genera of *Bacillus* (86.7%), *Geobacillus* (2.4%), *Paenibacillus* (1.0%), *Clostridium* (0.7%) and *Listeria* (0.5%), from Lasundra hot spring, Gujarat State, India.

Optimization of growth conditions for isolate

Optimization of Nutritional factors

The isolate *Bacillus subtilis* SHVSC04 displayed growth in the presence of various carbon sources with optimum growth in the presence of sucrose (Fig 2a). The growth pattern of isolates with different sucrose concentrations revealed that the growth of *Bacillus subtilis* SHVSC04 increases with the rise in sucrose concentration, while above 4% sucrose, the growth of isolate declines (Fig 2b). The isolate also showed growth in the presence of different nitrogen sources with optimum growth in the presence of yeast extract (Fig 2c). The growth pattern of isolate with different Yeast extract concentrations revealed that the growth of *Bacillus subtilis* SHVSC04 is highly effective in the presence of 2% Yeast extract. (Fig 2d). The growth of isolate also induced in the range of 1 to 5 % salt concentration, with significant growth at 2% (w/v) NaCl (Fig 2e).

Optimization of Environmental factors

The maximum growth of *Bacillus subtilis* SHVSC04 was obtained at 50°C; consequently, a 27% decrease in growth at 60°C and a 53 % decrease at 70°C reveal the thermotolerant nature of an isolate (Fig 3a). The isolate grows prominently at pH 6, 7 and 8, with maximum growth at pH 7; however, the growth of isolate declines by 32 % at pH 8 (Fig 3b).

Effect of nutritional and environmental factors on lipase production

The parameters were optimized by single-factor analysis to obtain the maximum extracellular lipase from *Bacillus subtilis* SHVSC04 which, are greatly influenced by nutritional factors including - carbon-nitrogen source, salt concentration, and physicochemical parameters, such as temperature and pH.

Effect of substrate

Amongst different substrates, tributyrin was found to be a superior substrate as compared to others, with the highest enzyme activity of 15.65U/ml, shown in Fig 4a. The optimal tributyrin substrate was recorded to be 2% with the

maximum enzyme activity of 19.55 U/ml, shown in Fig 4b. Wu HS and Tsai MJ, 2004 also reported tributyrin as the best substrate for lipase. *Bacillus firmus*, yielded optimum lipase enzyme activity at 1 % and can tolerate unto 5% tributyrin substrate (Patel et al., 2019). Patel et al., 2014 reported maximum lipase activity by *Pseudomonas sp*. DMVR46 at 1% concentration of tributyrin. A decrease in enzyme activity with an increase in tributyrin supplement in the media proposes the presence of substrate-level inhibition of lipase enzyme.

Effect of incubation period

The isolate produced a significant amount of lipase in tributyrin media on the 4th day (after 72 h) with maximum lipase activity (15.67 U/ml), whereas it attained maximum cell density after 48 hours (A600 nm = 1.02) of incubation. The decrease in cell density after 2nd day of incubation suggests the autolysis of cells. However, lipase production decreases approximately 20 % after continued incubation to 120 h, which might be due to degradation of the enzyme, as shown in Fig 4c. Bharathi et al., 2018 reported 48 h as the optimum incubation period for maximum growth as well as the highest lipase production from different bacterial strains. Krishnaveni 2013, accounted for 48 h incubation time for lipase production from *Staphylococcus aureus* MTCC 10787 from Tamilnadu, India. On disparity, optimization studies of *Bacillus methylotrophicus* PS3 by Sharma et al., 2017 reported 60 h of the optimum incubation period for maximum lipase production.

Effect of carbon source

Among the various carbon sources - glucose, dextrose, sucrose, lactose, maltose, and mannitol -supplemented in the lipase production media, sucrose was found to be the most effective at inducing the lipase production with the enzyme activity of 16.76 U/ml followed by glucose, mannitol, and maltose. However, dextrose and lactose depicted an inhibitory effect on lipase production shown in Fig 2a, which could be due to catabolite repression through readily available carbon sources in the medium (Patel et al., 2014). Furthermore, sucrose concentration was also optimized with maximum lipase activity at 3% sucrose (19.08 U/ml) and minimum lipase at 7% (15.69 U/ml), as revealed in Fig 2b. Similarly, Bharathi et al., 2018, also reported the highest lipase production by different bacterial strains in media that consists of 3% sucrose as a carbon source. Mazhar et al., 2017 also investigated that Bacillus *subtilis* PCSIRNL-39 utilizes sucrose to stimulate the gene expression for the production of high lipase content. While Veerabagu et al., 2013 reported the inhibitory effect of sucrose on lipase production in *Pseudomonas gessardi*.

Effect of nitrogen source

Among the various organic and inorganic nitrogen sources tested as additives, supplementing yeast extract yielded a significant amount of lipase, denoted by highest specific activity (16.29 U/ml), followed by peptone, ammonium nitrate, and ammonium phosphate. In contrast, ammonium sulfate (7.34U/ml) and urea (6.2 U/ml) revealed an inhibitory effect on lipase production, shown in Fig 2c. Furthermore, yeast extract concentration was optimized with maximum lipase activity at 3% yeast extract (18.97 U/ml) and minimum lipase at 7% (15.69 U/ml), as revealed in Fig 2d. On supplementing media with 0.5% yeast extract, *B. coagulance* BTS-3 produced maximum lipase (Kumar et al., 2005). Sidhu et al., 1998, and Kamini et al., 2000 also reported the highest lipase production under the influence of yeast extract in *Bacillus sp*. Bharathi et al.,2018 also reported 5% yeast extract as the best nitrogen source for maximum biomass and lipase production from different bacterial strains.

Effect of salt concentration

When supplementing media with 2% salt concentration, lipase production elevated with maximum enzyme activity (17.58 U/ml), while lipase production decreased, beyond 2% salt, shown in Fig 2e. Similarly, Selvin et al., 2009 also reported an increase in lipase production when the media was complemented with 2% salt in Nocardiopsis dassonvillei MAD08. Bora, L., & Bora, M., 2012 reported a maximum lipase production in *Bacillus sp*. isolated from hot spring of Arunachal Pradesh, India in the presence of 5 to 10% NaCl.

Effect of temperature

Temperature is an imperative factor for the production of extracellular bacterial enzymes. After 72 hours of incubation, maximum lipase production (16.42 U/ml) was obtained at 50°C, consequently, at 70°C, a 38 % decline in enzyme production, reveals the thermostable nature of the lipase (Fig 5a.) Likewise, Rathi et al. 2001 also reported an optimum temperature of 50°C for lipase production in *Burkholderia cepacia*. Kumar et al., 2005, showed the highest lipase production at 50°C in *Bacillus coagulance* BTS-3; Shreelatha et al., 2016 reported the highest lipase production from *Thermomyces lanuginosus* at 55°C; Sethi et al., 2016 declared that *Aspergillus*

terreus NCFT 4269.10 strain produced maximum lipase at 60 °C. The conformational changes and degradation of enzyme structure at high temperatures justify the decrease in enzyme activity.

Effect of pH

Lipase was active in a range of pH 6.0–8.0 with maximum production at pH 7.0 (17.25 U/ml) when examined for the range of pH 4.0 to 8.0. In contrast, lipase production reduced (11%) after incubation of 72 h, beyond pH 7, shown in Fig 5b. The decline in lipase production at higher pH reveals that enzyme conformation and transport across the cell membrane is affected by pH changes (Albert et al., 2002). Correspondingly, Rathi et al., 2001 also reported maximum lipase production in *B. cepacia* at pH 7, Gururaj et al., 2016 further reported a similar response of pH 7 as optimum pH for the lipase production in *Acinetobacter sp.* AU07. The detergent works in a pH range of 7 to 10.5, thus the *Bacillus subtilis* SHVSC04 lipase illustrates potential use in the laundry manufacturing.

Conclusion:-

Lipase producing bacterium was isolated and identified as *B. subtilis* SHVSC04 from Tuva Timba, a hot spring of Gujarat. The enzyme produced is stable at high temperatures, therefore can be used for the various industrial applications. Additionally, the enzyme can be economically produced in media supplemented with sucrose and yeast extract on ordinary commercial oils and shows the best activity at high temperatures and a higher pH range, presenting this enzyme as a potential biocatalyst for industrial applications. In the future, the research will focus on scale-up production and commercial utilization of lipase.

Acknowledgement:-

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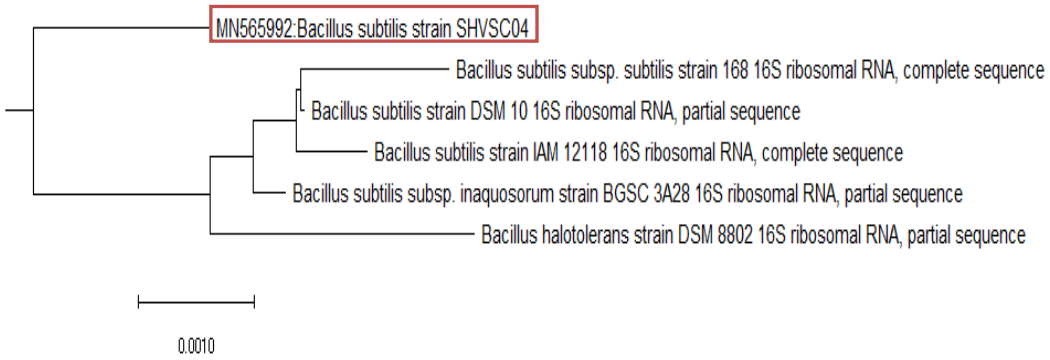
Table 1:-Biochemical characteristics of isolates; (+) positive result, (-) Negative result

Biochemical Test	*Bacillus subtilis* SHVSC04
Catalase test	+
Motility test	+
Gelatin hydrolysis	+
Nitrate reduction	-
Urea utilization	-
Phenylalanine determination	-
Slant	Alkaline
Butt	Alkaline
H_2S production	-
Gas production	-
Glucose	+ (acid production)
Lactose	-
Sucrose	-
Sorbitol	-
Starch hydrolysis test	+
Vogesproskauer's test	+
Methyl red test	+
Citrate utilization	Slightly utilized in Koser's Media
Indol	-
Cellulose	-
Oxidase	+

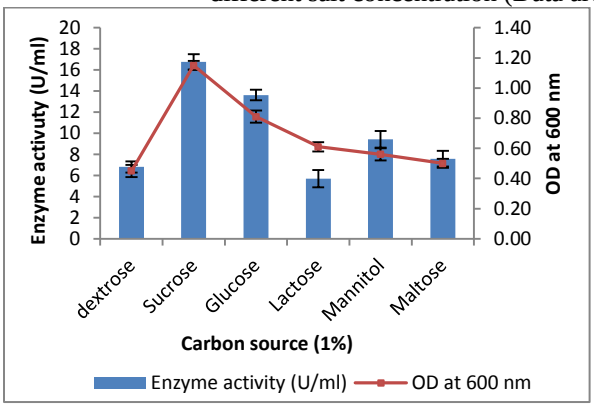
Fig 1:-(a) Gujarat Map; Hot springs of Gujarat; (b) Zone of hydrolysis formed by the lipolytic strain Bacillus *subtilis* SHVSC04; (c) The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.0010 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA X.

(a) (b)

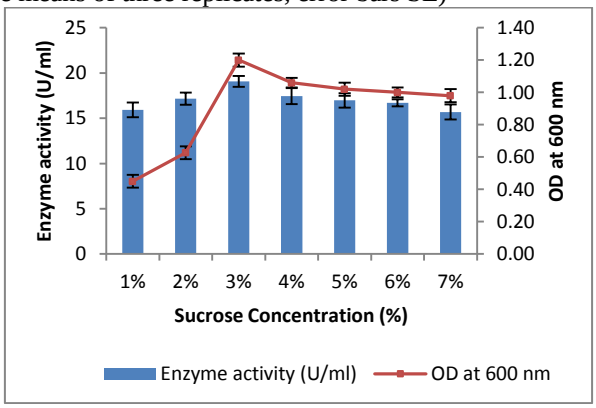


(c)

Fig 2:-Growth and lipase production profile of *Bacillus subtilis* SHVSC04 under (a) different Carbon source (b) different Sucrose concentrations (c) different Nitrogen source (d) different yeast extract concentrations (e) effect of different salt concentration (Data are means of three replicates, error bars SE)



(a)



(b)

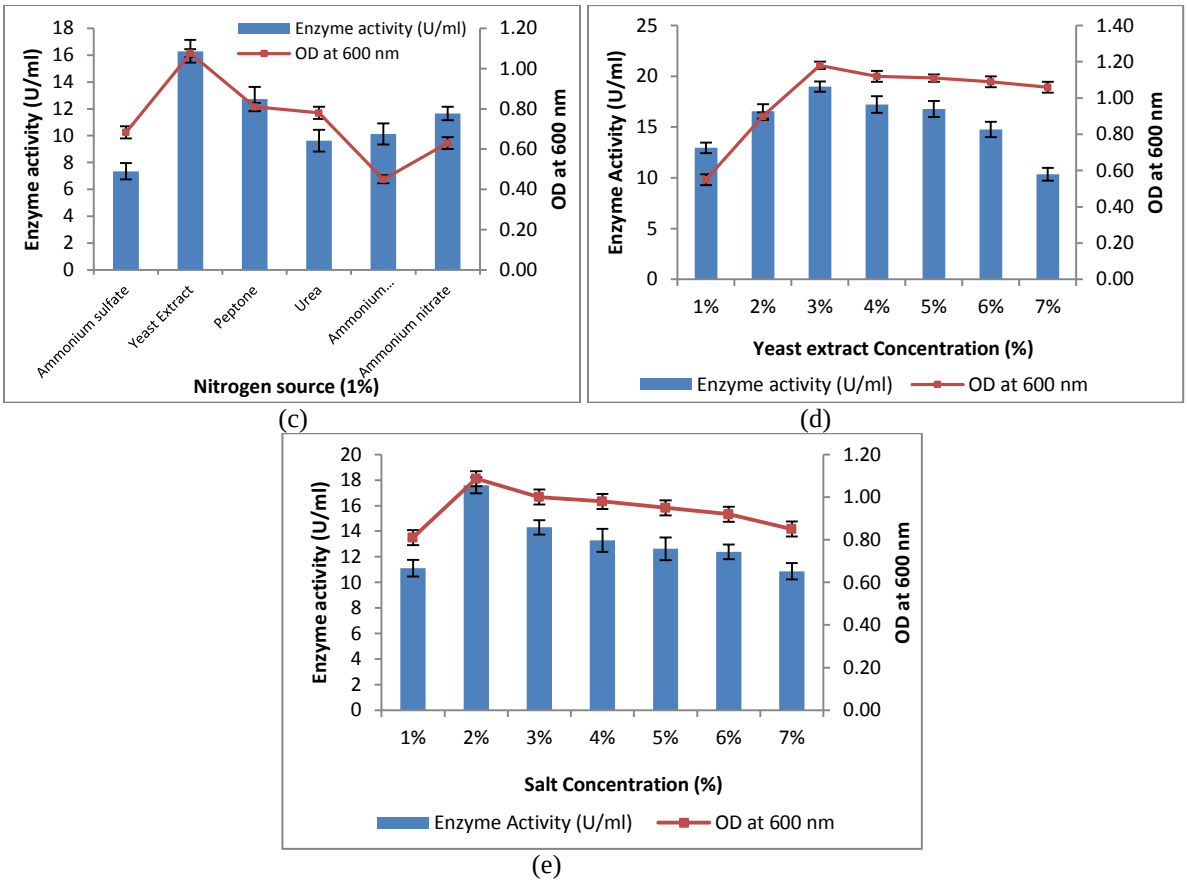


Fig 3:-Growth profile under: (a) effect of different temperature (b) effect of different pH (Data are means of three replicates, error bars SE)

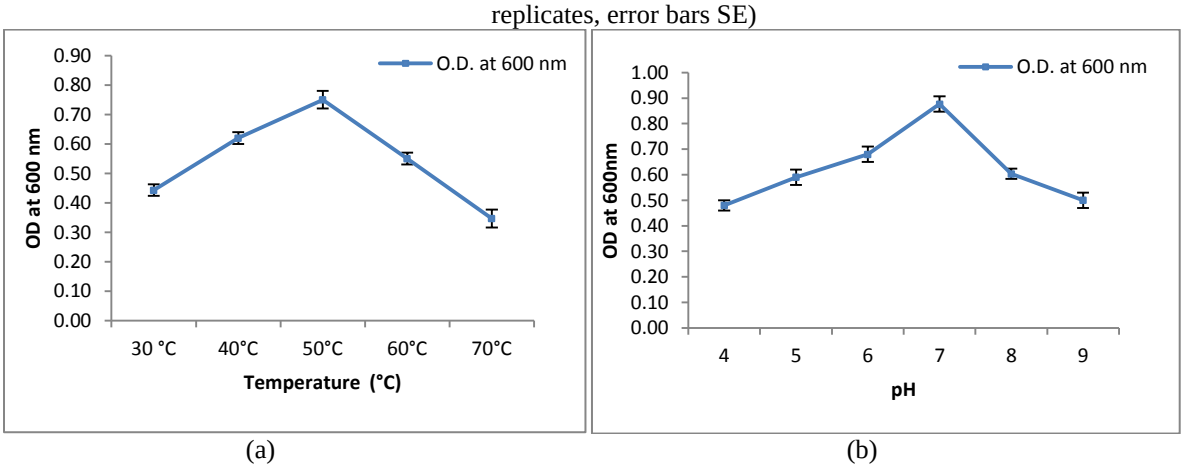
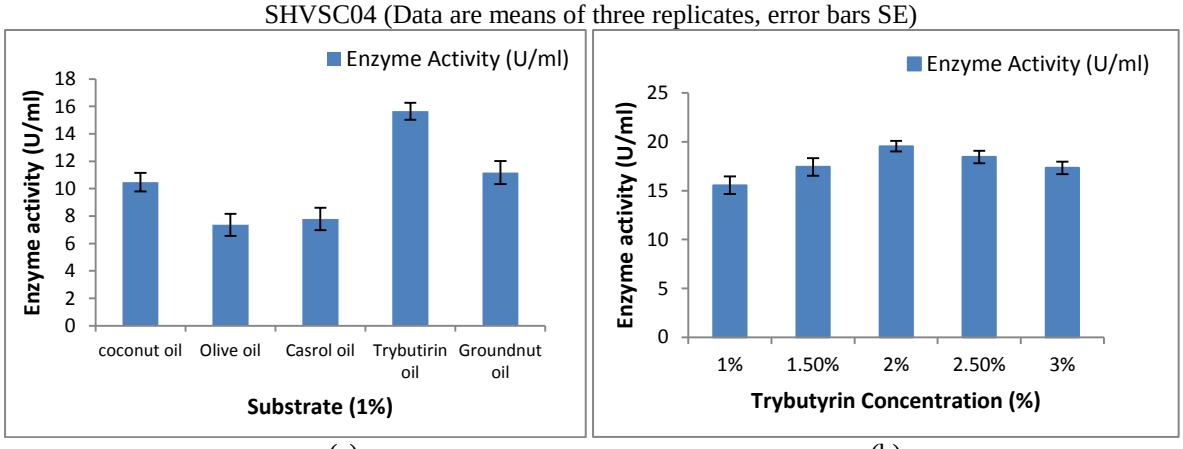


Fig 4:-Lipase production profile of *Bacillus subtilis* SHVSC04 under (a) presence of various substrates (b) effect of varying concentration of tributyrin (%) (c) Time course of growth and lipase production by *Bacillus subtilis* SHVSC04 (Data are means of three replicates, error bars SE)



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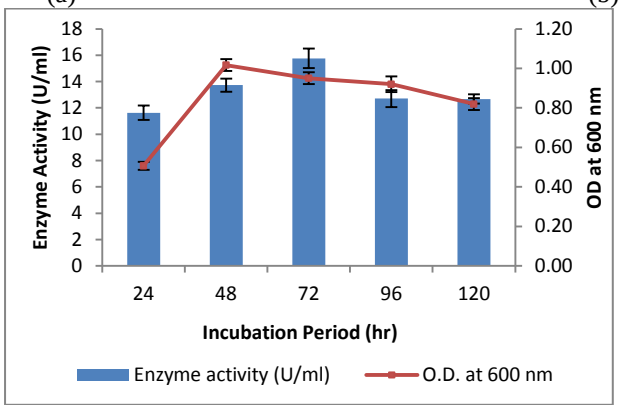
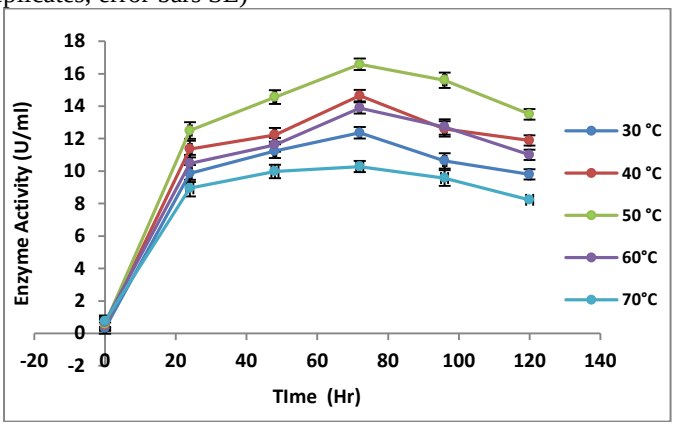
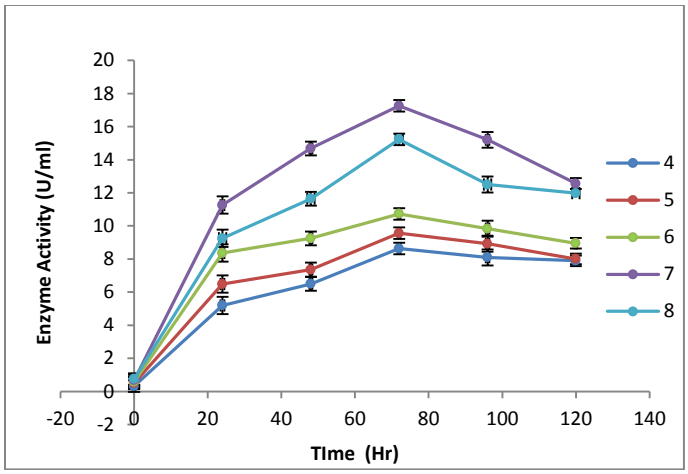


(c)

Fig 5:-Lipase production profile of *Bacillus subtilis* SHVSC04 under (a) Effect of temperature (b) Effect of pH (Data are means of three replicates, error bars SE)



(a)



(b)

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