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Research Article

SCREENING OF LEVAN PRODUCING BACTERIA FROM SOIL COLLECTED FROM JAGGERY FIELD

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ABSTRACT

Levan is an extracellular polysaccharide composed predominantly of D-fructose residues joined by glycosidic bonds. At present, there are many polysaccharides in the market primarily of plant origin. But microbial levans are more preferred as dietary polysaccharide due to its mult-idimensional application as anti-lipidemic, anti-cholesterimic, anti-cancerous and as prebiotic. In this context, in present study twelve levan producing isolates were screened out from jaggery field. Iosolate SJ8 was the highest levan producer which was studied for cultural, morphological and biochemical characterization. It was identified as *Bacillus subtilis* (KC243314). The optimized fermentation conditions for levan production were pH 7, temperature 30^0 C,100rpm and 48hrs fermentation period. At these optimized conditions the maximum levan production was 30.6mg/ml. Sugar composition of the polymer was confirmed by analytical methods and TLC. Result proved the presence of fructose as the main sugar in the polymer.

Keywords: Levan, *Bacillus subtilis* (KC243314), polymer, fructose.

INTRODUCTION

Levan is a biopolymer formed by β-(2,6) linkage having β-(1,2) linkages on its branch points and contains long chain of fructose units in its chemical structure . It develops in the course of a trans-glycosylation reaction with the participation of the levansucrase [EC 2.4.1.10][1]. Studies to re-search levan producers are related to its multifaceted applications in the food, cosmetic, pharmaceutical and medicine industries. Levan can be used as an emulsifier, formulation aid, stabilizing thickener, surface-finishing agent, encapsulating agent as well as a carrier for colours and flavours in the food industry[2]. In pharmaceuticals levan is a non-digestible food fibre[3] and, therefore, it can be treated as a component of prebiotic nature. Levan can also be utilised to manufacture DFA lV[di-D-fructose dianhydride]. DFA lV is characterised by half the sweetness of saccharose, cyclical structure which guarantees its stability and, therefore, similarly to other DFAs, it can find application as a sweetener for diabetics[4]. In medicine, it can be used as a plasma substitute, drug activity prolonger[5], anti-obesity and lipid-lowering agent[6], osmoregulator, cryoprotector, or antitumor agent[7]. The application potential of this biopolymer in the entire sectors is stimulating an intense and constant search aiming for a better new options so that we can get better alternative. In this context the present work was aimed to screen levan producers from soil, which is the rich source of various bacterial species.

MATERIALS AND METHODS

Sample collection

Levan producers grow very well in sucrose containing medium, therefore soil from jaggery preparation fields were selected as samples for this study. Soil samples were collected from the different areas where jaggery was prepared from sugarcane.

Enrichment of bacteria

Enrichment of bacteria was carried in sucrose broth containing 5% sucrose. For this 1% soil

sample was inoculated to 10ml sucrose containing broth. Tubes were incubated at 30° C for 48 hrs.

Screening of Levan producing bacteria

Ten fold serial dilutions of the tube showing turbidity were prepared. 1ml of diluted sample was spread inoculated on 5% and 10% sucrose agar plates. Plates were incubated at 30^{0}C for 48 hrs. Organisms producing mucoid colonies were selected as levan producer. Cultural and morphological characters of colonies were studied and colonies were sub-cultured on nutrient agar slants and preserved for further studies.

Levan production

Inoculum preparation

Inoculum preparation was carried by inoculating active bacterial culture in 5 ml of inoculum medium containing [g/L] sucrose 100, yeast extract 2, $(NH_4)_2SO_4$ 1, KH_2PO_4 2, $MgSO_4$ 0.5. The inoculated medium was incubated at 30^{0}C for 24 hr.

Production medium

5% of inoculum was transferred aseptically to 50ml production medium in 250ml Erlenmeyer flask. The production medium had following composition [g/L] sucrose 100, yeast extract 2, $(NH_4)_2SO_4$ 3, KH_2PO_4 1, $MgSO_4$ 0.6, $MnSO_4$ 0.2. The pH was adjusted to 7 before autoclaving. The inoculated production medium was incubated at 30^{0}C for 48 h on rotary shaker at 100rpm.

Extraction of Levan

After incubation the 10ml of fermentation broth was taken and centrifuged at 10000 rpm for 10 min. Cell pellet obtained was dried for biomass calculation and the clear supernatant was used for levan recovery.

Recovery and Determination of Levan

The supernatant obtained was then used to precipitate levan by adding alcohol. Four volumes of chilled aqueous ethanol [70%] was added to it. The tube was inverted several times for proper mixing. The precipitate formed was allowed to settle down at 4^{0}C for 24hrs. The supernatant was discarded and precipitate was collected. The precipitate was washed twice with aqueous ethanol and transferred to vial. It was dried at 60^{0}C[8,9] and dry weight was measured.

Biochemical characterization

Highest levan producer was characterized biochemically by inoculating in different biochemical media, sugar fermentation media and enzyme production media.

Identification by 16S rRNA sequencing

The high levan producing strain was identified by 16S rRNA sequencing from ARI Pune. Genomic DNA of isolate was isolated using Gene Elute Genomic DNA Isolation Kit (Sigma, USA) used as template for PCR. Each reaction mixture contained approximately 10ng of DNA; 2.5mM $MgCl_2$; 1x PCR buffer [Bangalore Genei, Bangalore, India]; 200µM each dCTP, dGTP, dAPT, and dTTP; 2 pmol of each, forward and reverse primer; and 1 U of Taq DNA polymerase[(Bangalore Genei, Bangalore, India] in a final volume of 20µl. FDD2 and RPP2 primers were used to amplify almost entire 16S rRNA gene, as described previously [Rawlings 1995]. The PCR was performed using the Eppendorf Gradient Mastercycler system with a cycle of 94^0 C for 5 min; 30 cycles of 94^0 C, 60^{0}C and 72^0 C for 1min each; and final extension at 72^0 C for 10min, and the mixture was held at 4^0 C. The PCR product was precipitated using polyethylene glycol (PEG 6000, 8.5%), washed thrice using 70% ethanol and dissolved in Tris-HCL [10mM, pH 8]. Samples were run on ABI Prism 3100 Genetic Analyser. The sequencing output was analyzed using the accompanying DNA Sequence Analyzer computer software [Applied Biosystems]. The sequence was compared with NCBI GeneBank entries by using the BLAST algorithm.

Analytical studies

The levan powder obtained was dissolved in deionized water.Total carbohydrate estimation in levan solution was carried by phenol sulphuric acid method[10]. 2ml of 0.1N HCL was mixed with 2ml of levan solution and boiled at 100^{0}C for 60min. The acid hydrolyzate was analyzed for fructose and glucose estimation. Fructose estimation was carried by Resorcinol method[11] and glucose was estimated by Somogyi method[12].

TLC of levan

Hydrolyzed samples were applied to Silica Gel plates saturated with solvent 1 Butanol: 2 propanol: water: acetic acid [7:5:4:2]. Standards of glucose [Hi media Pvt. Ltd.] and fructose were run and plates were sprayed with 5% sulfuric acid in methanol, air dried and then heated at 110^0 C until spots appeared.

Optimization of fermentation conditions on levan production

Highest levan producer was used for optimization of fermentation conditions for

levan production. The parameters studied were pH, temperature, agitation and incubation period.

Effect of pH on levan production
Levan production was carried at pH 4, 5, 6, 7, 8, and 9. pH of the medium was adjusted by using 0.1N HCl and 0.1N NaOH. The 50ml of production medium was inoculated with 24 hrs old culture and incubated at 30^0C for 48hrs. Levan and biomass was measured.

Effect of temperature on levan production
50ml production medium was inoculated and incubated at different temperature for 48hrs. The temperatures used were 10 ^{0}C, 20 ^{0}C, 30 ^{0}C, 40 ^{0}C, 50 ^{0}C and 60 ^{0}C. After this the levan was precipitated, dried and weighed. Similarly the effect of temperature on biomass was studied.

Effect of agitation
24hrs old inoculum was inoculated in 50ml production medium and incubated at 30^0C for 48hrs at agitation speed of 50rpm, 100rpm, 150rpm, 200rpm and 250rpm, one flask was kept at stationary. After completion of fermentation levan was precipitated, dried till constant weight. Also the effect of agitation on biomass was studied.

Effect of fermentation period
24hrs old culture of inoculum was inoculated in 50ml production medium and incubated at 30^0C and 100rpm for 24hrs, 48hrs, 72hrs, 96hrs and 120hrs of fermentation period. After completion of fermentation period biomass was measured; levan was precipitated, dried and weighed.

RESULTS AND DISCUSSION
12 isolates producing mucoid colonies on 10% sucrose agar plates were selected as levan producer. Levan producers hydrolyze sucrose to fructose and polymerize to levan by levansucrase giving mucoid colonies. All these isolates were Gram positive produced large, pale coloured colonies with entire margin [Table 1].Similar type of colonies on sucrose agar plates were screened out by Vaidya and Prasad [2012] and Ghale et al [2007] as levan producers. Levan production from these selected isolates was shown in fig 1. Maximum levan production i.e.25.2mg/ml was given by isolate SJ8, while The isolates SJ3, SJ5 and SJ7 produced 14mg/ml, 14.9mg/ml and 12.4mg/ml respectively. The low levan production was shown by isolates SJ4, SJ6 and SJ10. The highest levan producer was selected for optimization of fermentation conditions. This isolate was identified by biochemical characterization and 16S rRNA gene sequencing.

Analytical studies
Total carbohydrate content of levan produced [Fig. 2] by majority of the isolates was more than 400(µg/ml). Maximum carbohydrate content was found in levan [650 µg/ml] produced by isolate SJ8.
Levan produced by 12 isolates was hydrolyzed and hydrolyzate was analyzed for fructose and glucose content. Hydrolyzate from isolate SJ8 showed maximum fructose concentration i.e. 460µg/ml. Hydrolyzate from 8 isolates showed fructose content above 200µg/ml [Fig. 2].Only the presence of fructose and negligible presence of glucose confirmed the presence of fructose polymer.
The organism was caesinase, gelatinase, catalase and oxidase positive [Table 2]. It fermented sugars glucose, arabinose and xylose with acid production only. The organism showed temperature tolerance upto 80^0 C [Table 3].

TLC of levan
Sugar components of levan were identified by TLC analysis levan hydrolyzate. The Rf value of acid hydrolyzed levan from *Bacillus subtilis* (KC243314) was identical to that of fructose under laboratory conditions. This result indicated that levans were composed solely of fructose.

Effect of fermentation conditions on levan production
Effect of pH
The effect of initial pH of fermentation medium on levan production was shown in Fig 4. Levan production was found negligible at pH 4, then increased markedly at pH 5 to 22.3 g/L, at pH 6 it became 28.0g/L and finally reached to maximum at pH 7 i.e. 30.6 g/L. At pH 8 the production decreased markedly and became very low at pH 9. Similarly initially at pH 4 biomass found to be negligible i.e. 0.9 g/L, increased gradually and reached maximum to 10.65 g/L at pH 7 within 48 hrs. Below pH 5 levan production was negligible and beyond pH 8 it was low. This showed that extremes of acidic and alkaline conditions inhibited the levan production. Similarly biomass was also negligible below pH 5 and above pH 8 due to unfavorable ionic concentration for growth of organism. Thus levansucrase enzyme catalyzing levan formation was found active in broad range of pH 5 to pH 8. Similar results were reported by Abou-taleb et al [2014], showing maximum levan production at pH 7.8 [18.82g/L] from *Bacillus sp.*V8 strain.

Effect of temperature on levan production

At initial temperature of 10^0 C levan production was low (12.4 g/L), as the temperature increased levan production reached to 26.1g/L at 20^0 C as shown in Fig 5. Further increase in temperature to 30^0 C leads to highest levan production to i.e. 30.6 g/L, it became 25.0 g/L at 40^0 C, finally reached to 15.7g/L and 3.4g/L at 50^0 C and 60^0 C respectively. The highest biomass was 12.50g/L at 30^0 C. These results are in agreement with Abdeh –Fattah et al who reported the conversion of fructose to levan by *Bacillus subtilis* NRC33a was highest at 30^0 C. The levanforming activity of levansucrase showed activity in temperature range of 10^0 C to 50^0 C which was a broad activity zone, in between psychrophilic to thermophilic range.

Effect of agitation

Effect of agitation at 100g/L sucrose, 30^0 C for 48hrs was studied on the levan formation from *Bacillus subtilis* (KC243314). One flask which was kept at stationary [without agitation] gave 14.9 g/L levan production. Levan production was 28.0 g/L at 50rpm [Fig 6]. Further increase in agitation to100rpm increased levan production to 30.6 g/L which was the highest production. At 150rpm it became 20.0 g/L and finally reached to 14.0 g/L and 9.5g/L at 200rpm and 250rpm respectively. Biomass showed direct proportionality with levan production and it was highest at 100rpm i.e. 15.0g/L and lowest 1.2 g/L at 250rpm. At stationary biomass became 3.78 g/L. Thus mild aeration affects positively on levan production. Santose et al [2013] got maximum levan production of 34g/L at 150rpm with *Bacillus subtilis* NATTO.

Effect of fermentation period on levan production

Effect of incubation period on levan production from *Bacillus subtilis* (KC243314) was studied for 24hr, 48hr, 72hr, 96hr and 120hr as shown in Fig 7. After 24hrs the biomass and levan production became 4.54g/L and 11.5g/L respectively. The biomass and levan formation reached to maximum after 48hrs i.e. 9.5 g/L and 30.6g/ respectively. Once the levan formed in the fermentation broth remained as it is, there in no further increase in levan after 48hrs. But the biomass depleted later due to exhaustion of important nutrient factors. However Khadiga et al [2014] got maximum levan production after 96hrs with *Bacillus subtilis* sp.V8.

CONCLUSION

The current study aimed to screen out potent levan producer from natural source which is much valuable entity from medicinal and pharmaceutical point of view. In this study *Bacillus subtilis* (KC243314) was isolated produced 30.6g/L levan at pH 7, 10% sucrose concentration, at 30^0 C, 100rpm within 48hrs. By comparing the results with the literature available the isolate obtained in the present study was highly efficient *Bacillus subtilis* strain isolated from soil which produced very good quantity of levan at normal laboratory conditions. Further research in the optimization and purification of levan is in progress.

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Table 1: Cultural and morphological characterization of soil isolates incubated at 30^0C for 24hrs

Isolate	Size	Shape	Colour	Margin	Elevation	Opacity	Consistency	Gram's nature	Motility
1. SJ1	4mm	Irregular	Pale coloured	Smooth	Flat	Translucent	Mucoid	G+ve short rods	Motile
2.SJ2	3mm	Irregular	Pale coloured	Smooth	Flat	Translucent	Mucoid	G+ve short Rods	Non Motile
3.SJ3	1mm	Round	Pale coloured	Entire	Raised	Translucent	Mucoid	G+ve thick Rods	Motile
4.SJ4	3mm	Round	Pale coloured	Entire	Convex	Transparent	Mucoid	G+ve thick Rods	Motile
5. SJ5	2mm	Irregular	White	Smooth	Flat	Opaque	Mucoid	G+ve thick Rods	Non Motile
6.SJ6	2mm	Irregular	Pale coloured	Entire	Raised	Opaque	Mucoid	G+ve long Rods	Motile
7.SJ7	2mm	Round	Pale coloured	Entire	Raised	Opaque	Mucoid	G+ve rods	Non Motile
8. SJ8	4mm	irregular	White	irregular	Raised	Opaque	Mucoid	G+ve long Rods in chain	Motile
9. SJ9	3mm	Round	Pale yellow	Entire	Raised	Opaque	Mucoid	G+ve cocci	Non Motile
10.SJ10	Pin point	Round	Pale coloured	Entire	Convex	Opaque	Mucoid	G+ve cocci in bunch	Non Motile
11.SJ11	4mm	Irregular	Pale coloured	Smooth	Flat	Transluscent	Mucoid	G+ve rods	Motile
12.SJ12	2mm	Round	Pale coloured	Entire	Raised	Opaque	Mucoid	G+ve rods	Actively motile

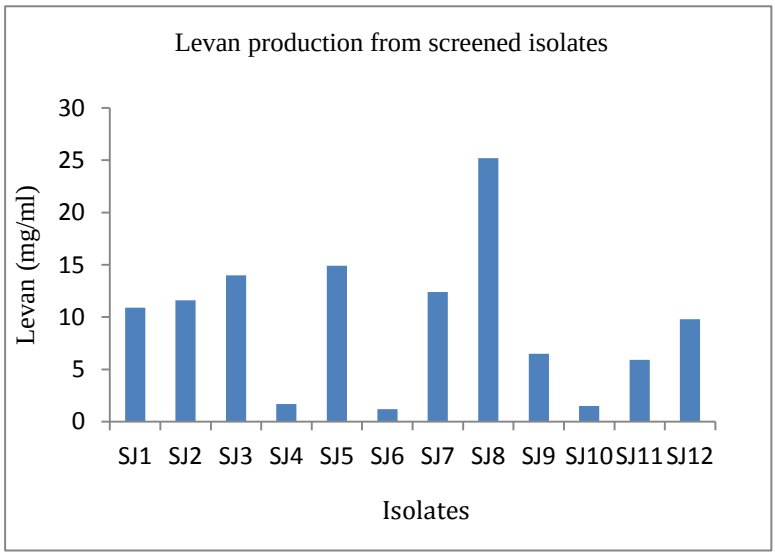

Fig. 1:

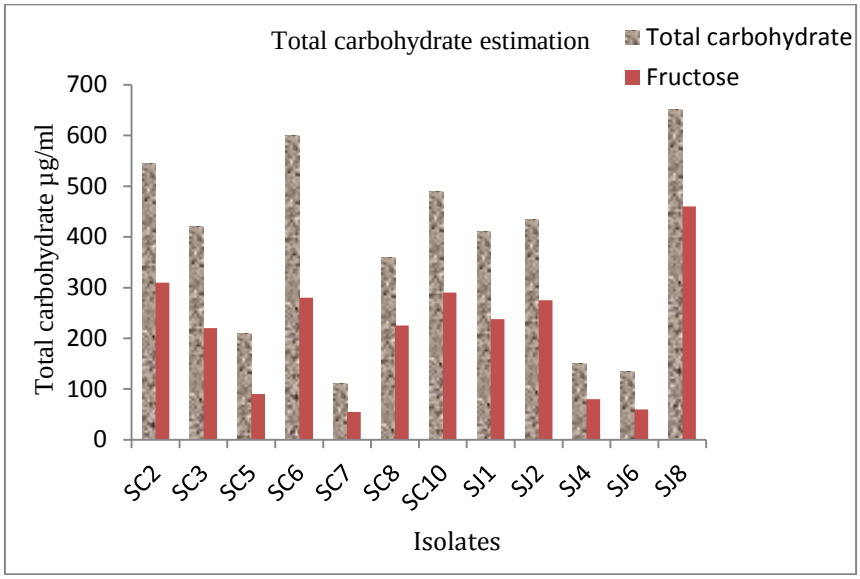

Fig. 2:

Table 2: Biochemical characterization of high levan producing isolate SJ8

Indole Test	-ve
Methyl red test	-ve
V.P. test	+ve
Citrate utilization	+ve
Glucose	A
Mannitol	A
Arabinose	A
Xylose	A
Caseinase	+ve
Gelatinase	+ve
Amylase	+ve
Nitrate reductase	+ve
Catalase	+ve
Oxidase	+ve

Legends: A: Acid, +ve: production, -ve: no production.

Table 3: Temperature and salt tolerance

Temperature tolerance	
20⁰ C	+
40⁰ C	+
60⁰ C	+
80⁰ C	-
Salt tolerance	
2%	+
5%	+
7%	+
10%	-

Legends: + Growth, - No growth

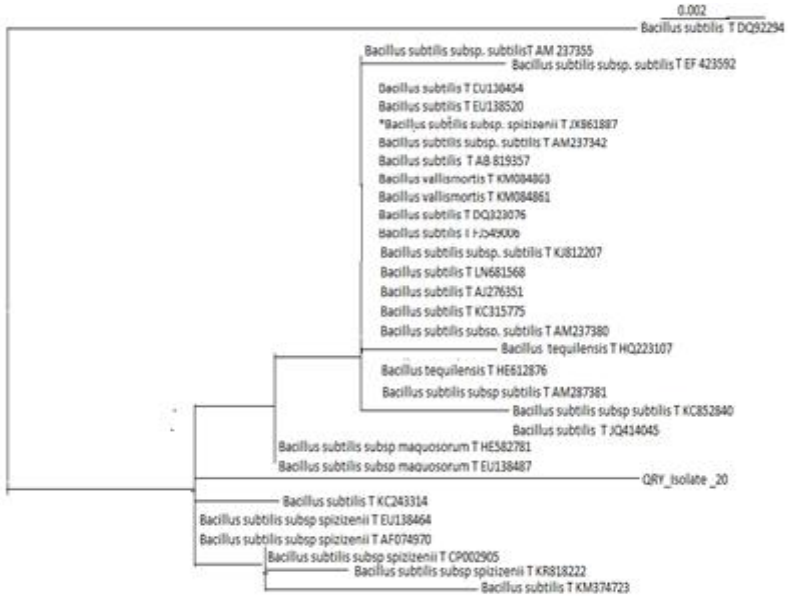

Fig. 3: The isolate SJ8 showed phylogenetic similarities (99%) with *Bacillus subtilis* and identified as *Bacillus subtilis* (KC243314)

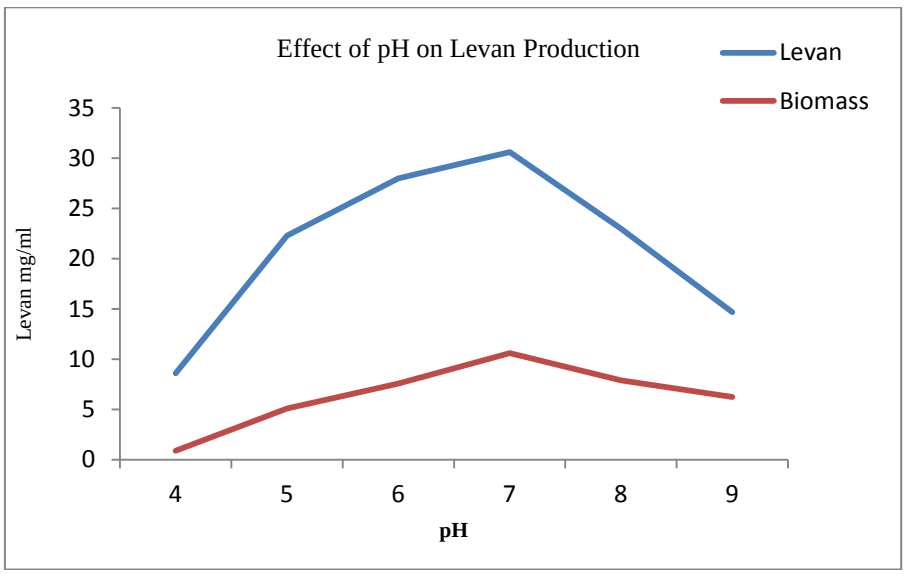

Fig. 4:

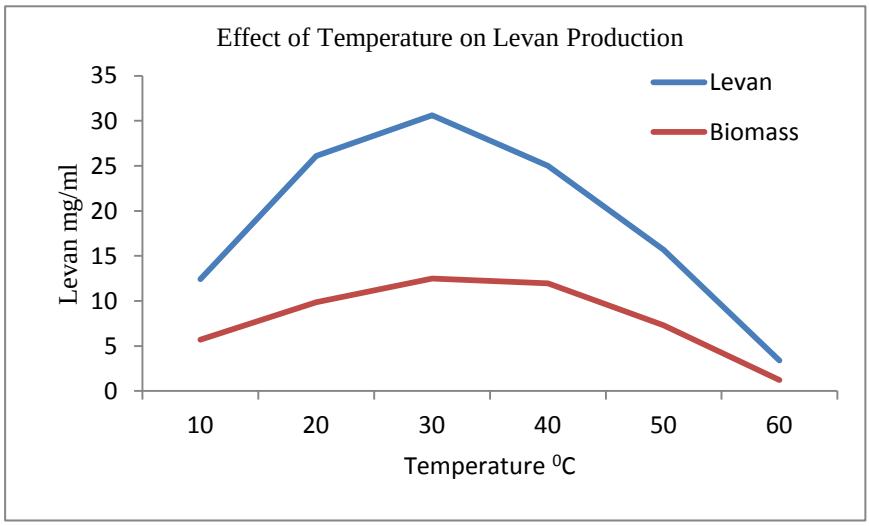

Fig. 5:

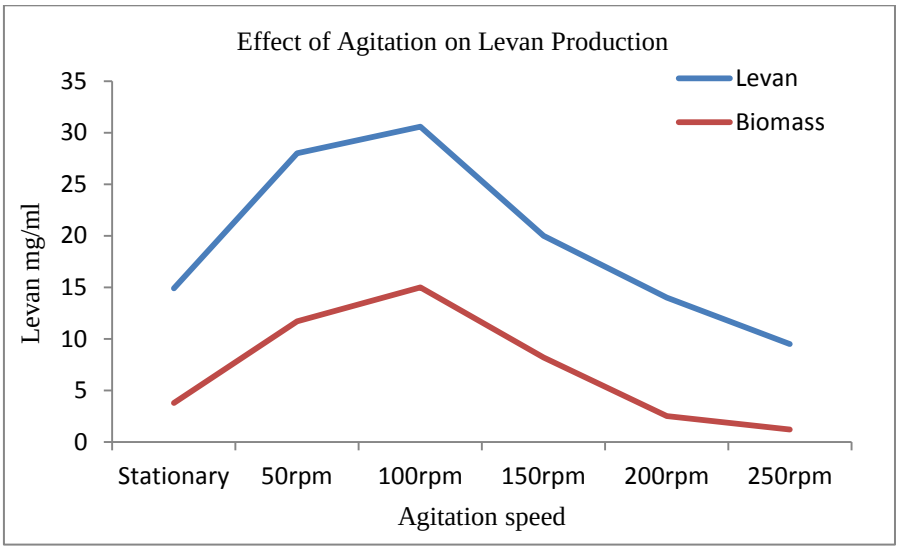

Fig. 6:

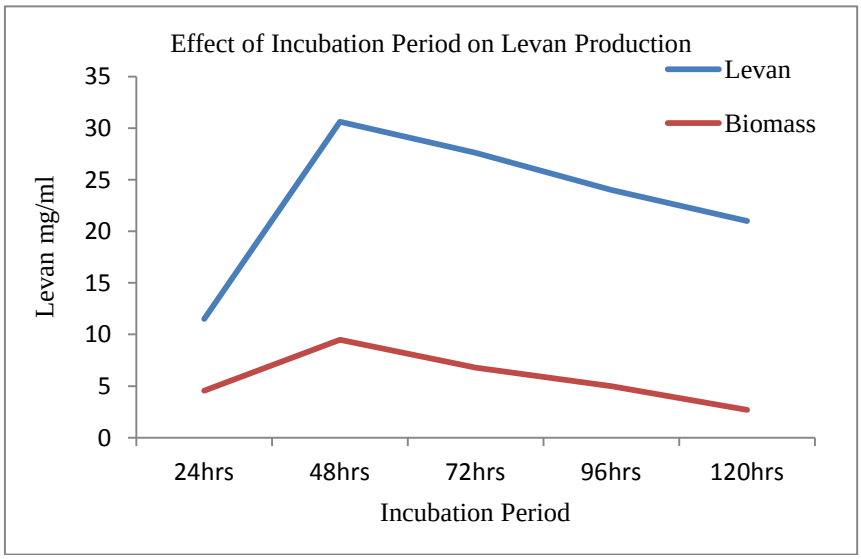

Fig. 7:

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