DEXTRAN PRODUCTION BY MICROBIAL BIOTRANSFORMATION OF SUGARCANE WASTE

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خلاصہ

قدرتی حیاتیاتی کثیر سالمیات نے کثیر المقاصد پولی سیکھرائیڈز (polysaccharides) کے طور پر دنیا بھر میں اہمیت حاصل کر لی ہے۔ پوری دنیا میں مختلف صنعتوں کی طرف سے قدرتی حیاتیاتی کثیر سالمیات کی طلب روز بروز بڑھ رہی ہے جو کہ جرثوں میں سے حاصل کیے گئے ایگزوپولی سیکھرائیڈز (exopolysaccharides) پر تحقیق کی نئی راہیں کھولتی ہے۔ موجودہ مطالعہ دیسی ذرائع سے ڈیکسٹران (dextran) پیدا کرنے والے خورد عضویوں (microorganisms) کی علیحدگی اور کثیر سالمہ کی پیداوار پر اثرانداز ہونے والے اہم پیرامیٹرز (parameters) کی پیش کارکردگی کی ایک کوشش ہے۔ متعدد کھانے کے نمونوں سے 17 جرثموں کی اسٹرینز (strains) علیحدہ کی گئیں۔ زیادہ ڈیکسٹران (dextran) پیدا کرنے والی اسٹرین (strain) کی شناخت X21 *Leuconostoc mesenteroides* کے طور پر ہوئی۔ یہ پایا گیا کہ X21 *L. mesenteroides* کثیر سالمہ کی زیادہ مقدار ساکن حالت میں 24 گھنٹوں میں 30°C اور زراسی pH basic یعنی کہ pH 7 پر پیدا کرتی ہے۔ نیوٹرینٹ میڈیم (nutrient medium) میں چینی کے ساتھ راب (molasses) کی شمولیت 2.28 گنا زیادہ حیاتیاتی کثیر سالمے کی پیداوار دیتی ہے۔ مجموعی کاربوہائیڈریٹس (carbohydrates) اور ریڈیوسنگ شوگر (reducing sugar) کے مواد کا تخمینہ انکشاف کرتا ہے کہ اسٹرین (strain) دستیاب کاربن کو موثر طریقے سے حیاتیاتی کثیر سالمہ میں بدل رہی ہے۔ پس یہ مطالعہ ڈیکسٹران (dextran) کی تجارتی پیداوار کے لیے راب کو سستے سبسٹریٹ (substrate) کے طور پر استعمال کرتے ہوئے ماحول دوست اور موثر لاگت کا عمل پیش کرتا ہے۔

Abstract

Natural biopolymers have gained worldwide importance as multipurpose polysaccharides. The demand for natural polymers by different industries around the world is increasing day by day, leading to new avenues in research on microbial exopolysaccharides (EPS). Current study attempted for the isolation of dextran producing microorganisms from indigenous resources and optimization of major parameters affecting polymer production. 17 bacterial strains were isolated from various food samples. Higher dextran producing strain identified as *Leuconostoc mesenteroides* X21. It was found that *L. mesenteroides* X21 produces higher quantity of biopolymer in static condition at 30 °C for 24 h in slightly basic condition i.e. at pH 7. Inclusion of molasses into nutrient medium along with sucrose resulted in 2.28 times higher biopolymer yield. The total carbohydrates and reducing sugar content estimated also revealed an efficient transfer of available carbon into biopolymer production by the strain. This study presents an eco-friendly and cost-effective process for commercial production of dextran utilizing molasses as cheap substrate.

Introduction

Biopolymers are found to commercially important because of their structural and functional diversity (Lule *et al*., 2016). Among many exopolysaccharides dextran, xanthan, chitosan, levan have extensive applications in industries. Owing to ease in isolation and production of dextran producing bacteria, it is being produced worldwide for vast majority of industrial applications (Lapasin and Pricl, 1995). Due to its comparative stability and noble solubility, dextran is generally used in many divisions of industries, such as pharmaceutical, food and chemical industries. Insoluble dextran could be used as matrix for immobilization of biomolecules. Dextran is used as blood-plasma volume expander and to cure hypovolemia. In chemical industries dextran is used as adjuvant, emulsifier, carrier and stabilizer. Crossed linked dextran known as sephadex is extensively used for separation and purification of many important biomolecules like proteins in research (Kotari *et al*., 2015). Commonly dextran is used as gelling, viscosifying, texturing, and emulsifying mediator in many food products (Naessens *et al*., 2005). It is also being used as thickening agent in jam and ice cream as it stops crystallization of sugar, enhance moisture retaining capacity and preserves flavor and appearance of the food stuffs (Qader *et al*., 2005).

Dextransucrase (EC. 2.4.1.5) is the key enzyme used for the biosynthesis of dextran. It is an extracellular enzyme classified in glycoside hydrolase family GH70. It has the ability to produce higher molecular weight dextran in the presence of sucrose, maltose and isomaltose (Hector *et al*., 2015). Dextransucrase catalyzes the

transfer of of glucosyl residues to dextran polymer as well as produces fructose as a by-product by utilizing sucrose as a carbon source (Parlek *et al*., 2013). Certain microbial strains are able to synthesize dextran of particular branching system and side chains. In other words, each dextran structure is characteristic of a specific dextransucrase (Dos *et al*., 1998). The most common strain for the production of dextran is *Leuconostic mensenteroides*, commonly found in vegetables fruits, milk, grass etc (Villani *et al*.,1997). It was discovered later that the polymer is produced by varieties of bacterial species such as *Leuconsotics, Streptococcus, Glucanobacte*r, *Acetobacte*r having variation in their structure and properties (Qader *et al*., 2005).

Aim of this research was to explore inexpensive carbon source such as molasses to achieve higher dextran production with lower manufacturing cost. Sugarcane molasses is known to be comparatively inexpensive and renewable substrate for higher dextran synthesis and are extensively been used as a replacement for sucrose in several industrial fermentation processes (Mosavi *et al*., 2010). In this study, *Leuconostoc mensenteroides* X21 was grown on raw material sucrose and sugar cane molasses. Fermentation characteristics and dextran production were studied in order to optimize the production process.

Materials and methods

Isolation and characterization of dextran producing bacterial species: To isolate dextran producing bacterial strain isolation medium reported by Qader *et al.* (2001) containing sucrose as main carbon source along with different essential growth components was prepared in broth and agar medium (Table 1). Various fruits and vegetables such as ridge gourd, cucumber, brinjal, okra, tomato, turnip, round gourd and water caltrop (singhara) were used as a source for the isolation of dextran producing bacterial strains. A small piece of each sample was inoculated in broth and incubated over night at 25 °C. After that broths were serially diluted to 10^{-6} and spread on agar plates containing screening medium reported by Sarwat *et al*., (2013). Plates were incubated overnight at 25 °C. Slimy shiny colonies were picked and restreaked for purification. Subsequently these colonies were checked for dextran production by the method of Ul Qader *et al*., (2001). Higher biopolymer producing strain was isolated, Gram stained and observed with a light microscope to check the purity. Different biochemical tests were performed for identification of bacterial strain according to "Bergey's Manual of Determinative Bacteriology" and strain was identified on the basis of morphological and biochemical characteristics (Holt J, 1994). Routine culture was refrigerated on Nutrient Agar slants whereas long term storage was done by preserving in 15% glycerol at – 20 °C.

Dextran production: To study optimal parameters for maximum dextran production, 2.5 ml of production medium was inoculated with loopful culture of selected strain and incubated at 30 °C for 24 h. After incubation inoculum was aseptically transferred to 22.5 ml of broth, flask was further incubated at 35 °C for 24 h. The precipitation of dextran was achieved after 24 h of incubation. The culture media was centrifuged at 4000 rpm for 10 mins. The pellet was separated from cell free filtrate (CFF) and dried to estimate weight of microbial biomass. For precipitation of dextran equal amount of chilled absolute ethanol was added to CFF with constant stirring and left it overnight at 4 °C. The precipitates of dextran appeared were retrieved by centrifugation of broth at 4000 rpm for 10 mins. The cell pellet obtained was collected on filter paper and dried to estimate microbial biomass. (Qader *et al*., 2005).

Estimation of sugar content: The supernatant which was obtained after the biomass separation was used for the analysis of reducing sugar content by Miller method (1959). Total carbohydrate content was also measured by applying carb test (Chow and Landhausser, 2004). The results obtained were compared with standard solution of glucose for the determination of reducing sugar and total carbohydrate present in cell free fluid.

Optimization of physicochemical parameters: Microbial strain isolated was studied at different physicochemical parameters such as medium composition, carbon source, time required for incubation, pH of nutrient medium, temperature suitable for polymer production, and static and shake flask fermentation conditions in order to achieve higher dextran yield.

Formulation of minimal nutrient medium: For dextran production media containing different concentrations of sucrose (2%,4%, 6% and 8%) along with different essential nutrient i.e. 0.5% yeast extract, 0.5% peptone 0.5% K_2HPO_4, 0.1 % $MgSO_4$, 0.5% $CaCl_2$ 0.1% NaCl and 0.1% $MnCl_2$ (Iqbal *et al*., 2015) were used (Table 1).

Effect of carbon source: Medium containing 2% sucrose, 0.5% yeast extract, 0.5% peptone 0.5% K_2HPO4, 0.1% $MgSO_4$, 0.5% $CaCl_2$, 0.1% NaCl and 0.1% $MnCl_2$ (Iqbal *et al*., 2015) in combination with varying concentration (0.5% to 10%) of molasses (1:1 diluted with distilled water) was used to study its effect on polymer production. Quantity of biomass and biopolymer produced was estimated after each trial Qader *et al*., (2005).

Effect of temperature on dextran production: Microbial strain was inoculated in production medium and incubated at various temperatures (25-40°C) for 24 h. Biomass and biopolymer were recovered, dried and weighed after each trail to estimate optimum temperature for higher yield.

Effect of time course on dextran production: To determine the optimum duration for dextran production, the culture was incubated for varying incubation time (6 h, 12 h, 24 h and 48 h). The amount of biomass and biopolymer produced after every trail was quantified as described previously.

Effect of pH on dextran production: To determine the optimum pH for biopolymer production, microorganism was incubated in nutrient medium with varying pH values (5, 6, 7, 8 and 9). All other parameters for growth were kept constant and the production of dextran was quantified.

Effect of agitation on biopolymer production: To determine the requirement of shaking by the culture during fermentation, static vs. shake flask technique was performed. One flask was incubated in static condition and the other one was incubated in agitation condition (80 rpm). All other factors were kept constant.

Results

Isolation and purification of dextran producing bacteria: 17 dextran producing bacterial strains were successfully isolated. Initially food samples were inoculated in isolation broth followed by streaking on agar medium with selective nutrient necessary for screening of dextran producing strains and the plates were incubated at 25 °C for about 24 h. Among all the various strains obtained, higher biopolymer producing strain was isolated from okra that shows slimy shiny transparent colonies on selective medium (Figure 1).

Biochemical characterization: Among various strains, 04 high biopolymer producing strains were subjected to biochemical testing and found to belong to genus *Leuconostoc*. Bacterial strain X21 selected for the study on the basis of its capacity of higher biopolymer production. Biochemical characteristics of strain X21 shows close resemblance with *Leuconostoc mesenteroides* (Table 2).

Estimation of sugar content: The supernatant obtained after the biomass separation was used for the analysis of total carbohydrate and reducing sugar content. By comparing the results with standard curve the initial total carbohydrate was found to be 7.38% and final total carbohydrate content which was obtained after fermentation was found to be 4.45%. Similarly, reducing sugar content was also measured in comparison with standard curve. Initial reducing sugar content was found to be 9.5% whereas final reducing sugar content was estimated about 2.862%.

Optimization of physicochemical parameters

Effect of different concentration of substrate on dextran production: For dextran production, medium containing different concentrations of sucrose was prepared. It was observed that sucrose concentration affects the production of dextran (Figure 2A). The results show that the growth of bacterial strain *Leuconostoc mesenteroides* X21 gradually decreases with the increase of substrate concentration. The maximum growth was observed in 2% sucrose.

Effect of carbon source on dextran production: For dextran production medium containing 2% sucrose in combination with different concentration of molasses ranging from 0.5% to 10% and 5% molasses (without sucrose) was used. The results show the production of biomass was affected by increase in substrate concentration (Figure 2B). Maximum growth was obtained by incubating the culture in fermentation medium containing 5% molasses in combination with 2% sucrose. Further increase in concentration of substrate shows inhibitory effect on dextran production.

Effect of temperature on dextran production: Figure 2C shows that minimum growth of *L. mesenteroides* X21 was obtained at 25 °C and the biomass increases significantly by the increase of temperature and maximum growth was observed at 35 °C thereby yield of dextran also affected by the change of temperature. Dextran production was low at 25 °C as lower biomass was obtained at this temperature, but the yield of dextran was increased gradually by the increase of temperature and maximum biopolymer production was attained at 30 °C.

Effect of time course on dextran production: The result shows maximum growth of *L. mesenteroides* X21 was achieved by incubating the culture for 24 h and further increase in time course did not show any increase in biomass (Figure 2D).

Effect of pH for incubation: The ability of *L. mesenteroides* X21 to produce dextran at differ range of pH was explored. The result shows maximum growth of bacteria was achieved by culturing at pH 7 (Figure 2E). Further increase in pH of fermentation medium inhibits the growth of bacterial isolate whereas pH 7.0 was also not found supportive.

Effect of agitation on biopolymer production: The ability of bacteria to produce polymer at static or shake flask condition was explored. The result shows that *L. mesenteroides* X21 grows well on static condition rather than agitation. (Figure 2F). Very little biomass and dextran was produced on agitation.

Table 1: Composition of Nutrient Medium for Dextran Production

S.No	Component	Isolation Media (gm/100ml)	Production Media (gm/100ml)
01	Sucrose	2	2
02	Nutrient broth	1.3	-
03	Yeast Extract	-	0.5
04	Peptone	-	0.5
05	K_2HPO_4	0.5	0.5
06	$MgSO_4$	0.1	0.1
07	$CaCl_2$	0.5	0.5
08	$MnCl_2$	0.1	0.1
09	NaCl	-	0.1
09	Molasses (1:1 Diluted with Distilled water)	-	(0.5-10)

Table 2: Result of Biochemical Characterization.

s.no	Test	Standard	X21
1.	Gram staining	Gram Positive cocci or rods.	Gram positive, long rod shape bacteria
2.	Colonial morphology	Circular mucoid colony having slimy appearance	Circular colonies of creamy color with regular ,margins having smooth appearance
3.	Catalase test	Negative	-- +
4.	Citrate utilization	Positive	+ + +
5.	Urease test	Positive or negative	++ -
6.	Indole test	Positive or negative	+++
7.	VP test	Positive or negative	+ + +
8.	Methyl red test	Positive	+++
9.	Starch hydrolysis test	Negative	---
10.	KOH test	Negative	---
11.	Motility	Non motile	---
12.	H_2S gas production	Negative	---
13.	Tween hydrolysis	Positive Or Negative	+++
14.	Nitrate test	Negative	+ + +
15	Sugar fermentation test: Glucose Maltose Lactose	+++	 +++ +++ +++

+++= Positive, - - + = Partial positive, + + - = Partial negative, - - - = Negatives

Table 3: Effect of Carbon Source on Dextran Production

Carbon source(molasses with 2 % sucrose)	Initial total Carbohydrate %	Final total Carbohydrate %	Initial Reducing sugar %	Final Reducing sugar %	Biomass (mg/ml)	Weight Of Polymer(mg /ml)
0.50%	5.67	3.69	5.38	2.82	870	200
1%	5.92	3.45	5.69	2.78	1221	345
2%	6.39	3.15	5.96	2.79	1530	370
3%	6.54	3.01	6.08	2.86	1685	452
4%	6.99	2.93	6.19	2.93	1721	497
5%	7.14	2.85	6.28	2.86	1785	550
6%	7.45	2.74	6.37	2.96	1624	430
7%	7.51	2.92	6.45	3.12	1572	357
8%	7.89	3.07	6.58	3.23	1446	300
9%	8.1	3.19	6.67	3.35	1350	248
10%	8.31	3.31	6.76	3.47	930	171
5% molasses only	7.45	3.25	5.95	3.01	870	284

Discussion

In this study 17 bacterial strains were isolated from a variety of fruits and vegetables. With the help of selective medium (Sarwat *et al*., 2013) dextran producing *Leuconostoc* species were isolated. Highest dextran yielding strain was isolated from okra and after performing biochemical tests it was identified as *Leuconostoc mesenteroides* X21. It has similar phenotypic and biochemical characteristics as reported by Holt J.(1994). Studies show that Leuconostoc species isolated from different food sources exhibit variation in dextran production which is due to the differences in their glucosidic linkages (Cote and Robyt, 1982; Zahnley and Smith, 1995; Sarwat *et al*., 2013).

It is found that dextran production by *L. mesenteroides* strains is greatly affected by culture conditions and medium components (Vedyashkina *et al*., 2005; Sarwat *et al*., 2008). Present study was conducted to utilize molasses as cheap substrate and to optimize dextran production by indegeniously isolated *Leuconostoc mesenteroides* X21. The effect of substrate concentration and carbon source on bacterial growth and dextran production was studied. Inititally 2% sucrose in presence of 0.5% molasses was used for culturing, however, upon further investigation, 5% molasses along with 2% sucrose was found optimum for both bacterial growth & biopolymer production. Similar results are reported in a study where 2% sucrose was optimum for higher dextran production (Fatma and Benmechernene, 2013). Dextran production decreases in presence of higher sucrose level possibly either due to osmotic stress which inhibits growth (Lule *et al*., 2016) or due to substrate inhibitory effect (Devi et a., 2014; Sarwat *et al*., 2008). However, some studies report higher concentration of substrate favored higher dextran production (Behravan *et al*., 2003; Chow and Landhausser 2004; Sarwat *et al*., 2008; Katina *et al*., 2009). When 5% molasses was used for fermentation in absence of sucrose, the production of both biopolymer and biomass were at suboptimal level suggesting that the combination of sucrose and molasses is required. Similar results were also reported in a study where combination of substrate in a medium supports higher dextran production as compared to medium containing single substrate for polymer production (Behravan *et al*., 2003).

L. mesenteroides X21 shows maximum dextran production in 24 h of fermentation which is in accordance of results reported by Iqbal *et al*., (2015). On contrary some studies found 12 to 16 h fermentation sufficient for maximum dextran production (Goyal at al., 1995; Santos *et al*., 2005; Lule *et al*., 2016). On further incubation of *L. mesenteroides* X21, the dextran concentration starts decreasing. It is reported that dextran is produced during exponential phase and early stationary phase of *Leuconostoc* spp. and its production becomes either stable in decline stage or reduced from initial amount (Qader *et al*., 2005; Viyayendra and Babu, 2008). Factors responsible for the reduction in biopolymer yield are possibly pH of medium, temperature, type of strain as well as biopolymer degrading enzymes like glycohydrolase (Gancel and Novel, 1994; Mozzi *et al*., 1994).

Temperature also influenced the production of dextran. This study found maximum dextran production at 35 °C whereas minimum growth was observed at 25 °C. This clearly indicates that *L. mesenteroides* X21 tolerates high temperature and as the biomass increases it results in more dextran production. These results are similar to a study where 35 °C was found optimum for higher dextran production (Santos *et al*., 2000). However other studies reported 25 to 27 °C suitable for optimum dextran production (Zedan *et al*., 1983: Siddiqui *et al*., 2013; Lule *et al*., 2016).

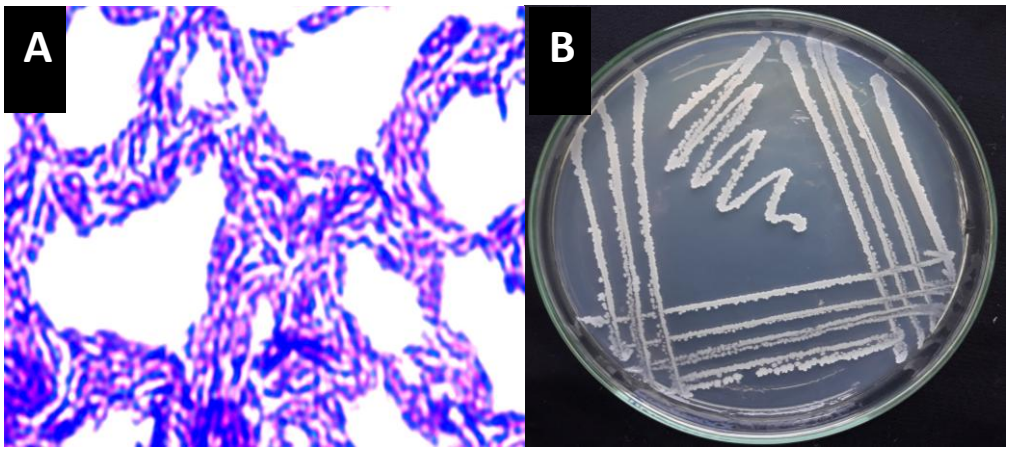

Fig.1. *Leuconostoc mesenteroides* X21: Microscopic Appearance (A), Growth on Production Medium (B)

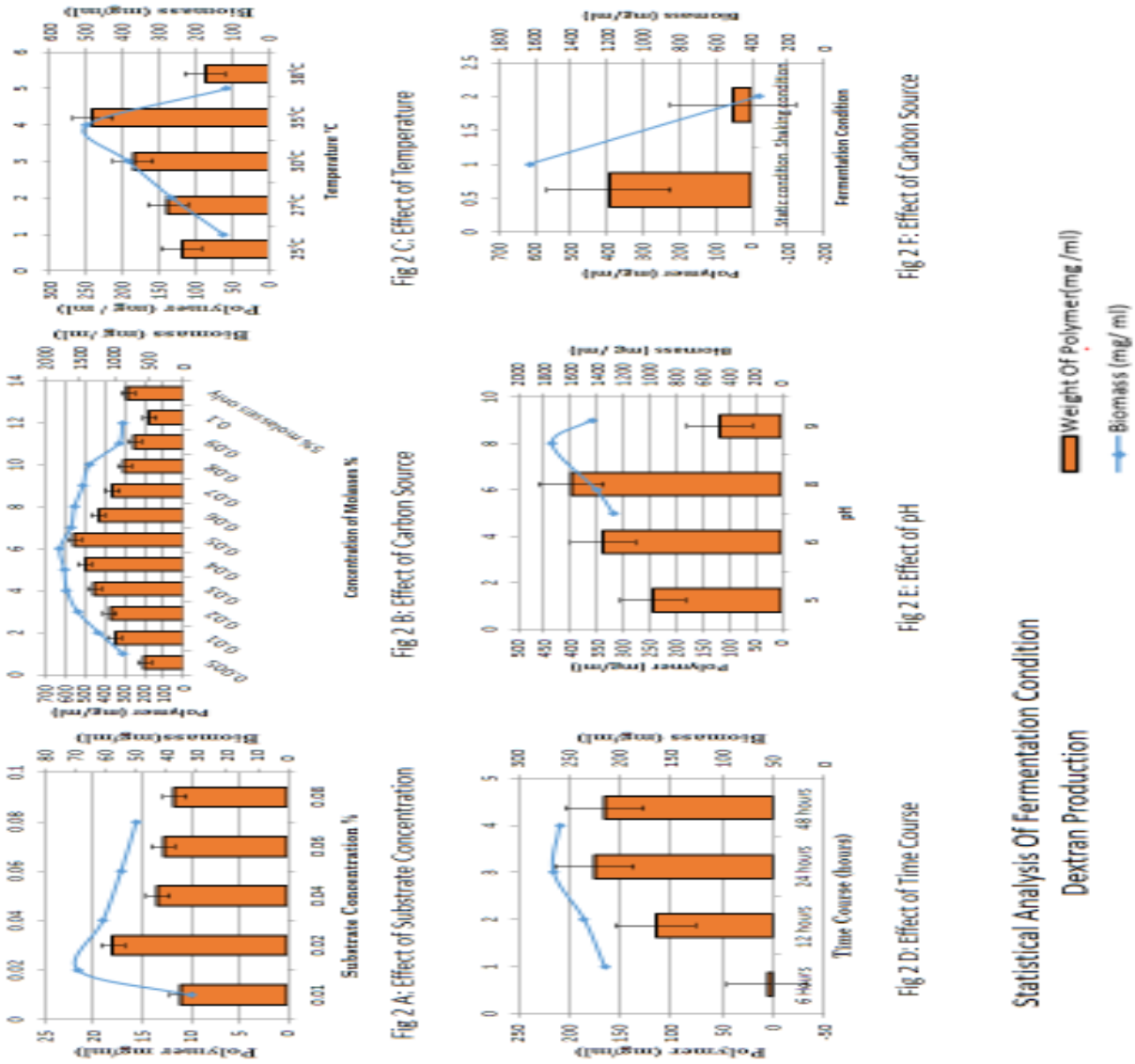

Fig.2. Statistical Analysis of Fermentation Condition of Dextran Production

It is reported that neutral pH values are suitable for optimim dextran production (Moosavi-nasab *et al*., 2010; Lule *et al*., 2016). However, in the present study maximum dextran production was observed at pH 7. These results are in agreement with studies where neutral pH were found optimum for higher dextran production (Zedan *et al*., 1983; Onilude *et al*., 2013). *L. mesenteroides* X21 grows well on static condition rather than agitation. Similar results are found in a research where static condition is preferred for dextran production. (Qader *et al*., 2008)

The fermentation conditions optimized in the present research requires further testing at pilot scale to ensure the commercial feasibility of the process. The current approach presents a novel, eco-friendly and cost-effective technique for commercial production of dextran by utilizing different industrial waste which can significantly contribute for the aim of biotechnology **"zero waste."**

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