Optimization and Statistical Evaluation of Medium Components Affecting Dextran and Dextransucrase Production by *Lactobacillus acidophilus* ST76480.01

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Abstract: On the basis of high enzyme activity a newly isolated strain was selected for dextran and dextransucrase production. Morphological, biochemical, and 16S rRNA sequencing analysis identified the strain as *Lactobacillus acidophilus* ST76480.01. It produced maximum dextran after 48 hours of incubation in the presence of sucrose as a carbon source, yeast extract and peptone as a nitrogen source at 30°C and pH 8.3. Present study reported statistical medium optimization for dextran and dextransucrase production for the strain comprising of Plackett-Burman. The increasing in sucrose concentration was significant for maximum dextran yield (4.24 mg/mL) and the highest dextransucrase activity (4.64 DSU/mL/hr) were obtained when 15% sucrose concentration was used while, the decreasing of K_2HPO_4 was significant for the highest dextransucrase activity (4.45 DSU/mL/hr) and (4.34 mg/mL) of dextran yield was obtained at (10 g/L) of K_2HPO_4.The structure of the polysaccharide dextran polymer was analyzed by ^{13}C nuclear magnetic resonance (NMR) spectroscopic techniques. It was confirmed that the insoluble dextran produced by *Lactobacillus acidophilus* ST76480.01 showed six major resonance peaks at 500 MHz: 104.245, 80.437, 76.192, 75.353, 63.258 and 59.739 ppm for linear linkages and the spectra of insoluble dextran was also found to contain minor peaks indicative of branching.

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1. Introduction

Dextransucrase is an extracellular enzyme produced by various species of the genera *Streptococus, Leuconostoc and Lactobacillus* involved in the synthesis reaction of dextran from sucrose (Barker and Ajongwen, 1991). It is a glucosyltransferase (E. C. 2.4.1.5) that catalyzes the transfer of glucosyl residues from sucrose (S) to dextran polymer and liberates fructose (F) according to the following equation (Hehre, 1951): $n\ S \rightarrow n\ F$ + dextran (glucose)*n*. Dextransucrase production is affected by several factors, such as temperature, pH, aeration and substrate concentration (sucrose). Generally the fermentation temperatures are about 23-26°C (Santos et al., 2000).

Dextran (C6H10O5)n is a high-molecular-mass (107 to 108 Da) glucan (Robyt, 1995). It is an extracellular bacterial polymer of D-glucopyranose with predominantly α- (1→6) linkage in the main chain and a variable amount of α-(1→2), α-(1→3), α-(1→4) branched linkages (Monsan et al., 2001). Dextran is commercially available, and it is used as drugs, especially as blood plasma volume expander. Dextran has also found industrial applications in food, pharmaceutical and chemical industries as adjuvant, emulsifier, carrier and stabilizer (Goulas et al., 2004). Cross-linked dextran is known as Sephadex, which is widely used for the separation and purification of protein. In food industry dextran is currently used as thickener for jam and ice cream (Naessens et al., 2005). It prevents crystallization of sugar, improves moisture retention, and maintains flavour and appearance of various food items (Purama and Goyal, 2008).

In the present work, we reported the isolation and biochemical identification of the lactic acid bacteria isolated from fermented vegetables. Further, the 16S rRNA was used to analyze the strain species. The basal medium composition for maximum production of dextran and dextransucrase activity has been optimized in 2 steps: (i) Studying the effect of different carbon, nitrogen sources, initial pH and fermentation temperature (ii) Plackett-Burman method used for selection of the most influential concentrations of medium components. The structure of the polysaccharide dextran polymer was also analyzed by ^{13}C nuclear magnetic resonance (NMR) spectroscopic techniques and thin layer chromatography (TLC).

2. Material and methods

Isolation and identification of *Lactobacillus* strain:

Lactobacillus strain was isolated from fermented vegetables on medium agar plates containing (g/L): Sucrose, 150.0; bacto-peptone, 5.0; yeast extract, 5.0; K_2HPO_4, 15.0; $MnCl_2.H_2O$, 0.01;

NaCl, 0.01; $CaCl_2$, 0.05; agar, 50; the pH was adjusted at 7.0 and autoclaved at 121ºC for 15 minutes. The plates were incubated at 25ºC for 48 hr. *Lactobacillus acidophilus* ST76480.01 was identified according to the Bergey's Manual of Determinative Bacteriology (Holt, 1994) and confirmed by 16S rRNA gene sequencing analysis using universal primers (16S-forward primer 5V AGGTCAAGTTTAT -3V and 16S-reverse primer 5V-CTATGGGACCA-3V) by using chain termination reaction as described by Hamasaki et al. (2003). The nucleotide sequences were analyzed with the BLAST database (Lipman, 1997). The sequence was deposited at GenBank with accession No ST76480.01. The strain was maintained on medium agar slants. These slants were kept at 4ºC for further experiments and were sub cultured monthly.

Medium composition: For dextran production, the culture was grown in the broth medium containing (g /L): Sucrose, 150.0; bacto-peptone, 5.0; yeast extract, 5.0; K_2HPO_4, 15.0; $MnCl_2.H_2O$, 0.01; NaCl, 0.01; $CaCl_2$, 0.05 and pH was adjusted to 7.0 before sterilization at 121ºC for 15 minutes.

Maintenance of culture and inoculum preparation: The isolated culture designated as *Lactobacillus acidophilus* ST76480.01 was streaked on medium agar slants and kept for incubation at 25±2ºC for 24 hr. These slants were kept at 4ºC for further experiments and were sub cultured monthly.

Cultivation conditions: For all optimization studies, 10.0 mL of overnight culture were inoculated in 250-mL Erlenmeyer flasks containing 90 mL sterile broth medium. Unless and otherwise mentioned all experiments were carried at 25ºC under static conditions for 48 hours. This 100 mL fermented broth was used for dextran production.

Time Course for dextransucrase and dextran production: To study the effect of time on dextran production, culture media were incubated for different time intervals (4-96 hours). Dextran production, dextransucrase activity, optical density and viscosity of fermented culture broth were determined.

Optimization of medium components for dextran production and dextransucrase activity

Selection of the most suitable carbon source: Sucrose of the broth cultivation medium was substituted with glucose, fructose, maltose, lactose, molasses and starch each at a time. Each carbon substrate was used as 10% (wt/vol) and all other medium components left unchanged.

Selection of the most suitable nitrogen source: Yeast extract and peptone of the broth medium were substituted with soybean, $(NH_4)_2SO_4$, NH_4Cl, whey, $NaNO_2$, casein and urea each at a time. Each nitrogen source was used as 1% (wt/vol) in equivalent nitrogen basis and all other medium components left unchanged.

Effect of pH: pH of the cultivation media was adjusted from 2 to 14 and they were incubated at 25°C for 48 hours.

Effect of temperature: It was studied in the temperature range of 15 to 45°C. For temperature optimization 100mL of fresh broths containing 10% sucrose was used.

Selection of the influential medium components for process modeling: In this experiment, seven factors of medium components were screened in eight combinations organized according to the Plackett-Burman design matrix shown in Table 1 and 2. For each variable, high (+1) and low (-1) levels were tested (Plackett and Burman, 1946; Rajendran et al., 2007).

Table 1: Different levels of the seven independent variables used in the Plackett-Burman design

Variable	High Level (+)	(O) Level	Low Level (-)
Sucrose	225.0	150.0	75.0
Peptone	7.5	5.0	2.5
Yeast extract	7.5	5.0	2.5
K_2HPO_4	22.5	15.0	7.5
$MnCl_2$	0.015	0.01	0.005
NaCl	0.015	0.01	0.005
$CaCl_2$	0.075	0.05	0.025

Table 2: Plackett-Burman design for seven variables

Trials (n)	Independent variables						
	1	**2**	**3**	**4**	**5**	**6**	**7**
1	+	+	+	-	+	-	-
2	+	+	-	+	-	-	+
3	+	-	+	-	-	+	+
4	-	+	-	-	+	+	+
5	+	-	-	+	+	+	-
6	-	-	+	+	+	-	+
7	-	+	+	+	-	+	-
8	-	-	-	-	-	-	-

The factors were sucrose, peptone, Yeast extract (YE), K_2HPO_4, $MnCl_2$, NaCl and $CaCl_2$ were analyzed as possible factors affecting production. The assays were performed in duplicate. The main effect of each factor was determined using the following equation: $E_{xi} = (\sum M_{i+} - \sum M_{i-})/N)$. Where E_{xi} is the variable main effect, M_{i+}, M_{i-} are the calculated results of the dextran production also for dextransucrase activity recorded by trial which contains positive levels and negative levels of independent variables (xi), respectively and N is the number of trials divided by 2. A main effect figure with a positive sign indicates that the positive level of this variable is nearer to optimum percentage of dextran production or dextransucrase activity while a negative sign

indicates that the negative level of this variable is nearer to optimum percentage of dextran production or dextransucrase activity. Using Microsoft Excell, statistical t-values for equal unpaired samples were calculated for the determination of variable significance (Al-Sarrani et al., 2006).

Preparation and purification of dextran:

The culture medium after 20 hours was precipitated using equal volume of chilled ethanol, shaken vigorously, centrifuged at 10,000 rpm for 15 minutes and the supernatant was decanted. For removal of impurities, precipitated dextran was dissolved in distilled water. The dextran slurry was again precipitated with equal volume of chilled ethanol. This procedure of re-dissolving, precipitation and washing was repeated thrice to remove cells debris. Purified dextran was dried under vacuum over calcium chloride at 30ºC. The dextran yield was calculated on dry weight basis (Qader et al., 2001).

Determination of viscosity:

Dextran solution (5%) was used as a stock solution for viscosity measurement at 25°C using an Ostwald Viscometer (Shamala and Prasad, 1995).

Dextransucrase standard assay:

Dextransucrase activity was determined by measuring the reducing sugar released from sucrose (Kobayashi and Matsuda, 1974). Units of dextransucrase activity were represented in DSU/mL/hr. "One unit of enzyme activity was defined as the enzyme quantity that converts 1.0 milligram of sucrose into fructose and dextran in 1.0 hour under standard conditions" (Lopez and Monsan, 1980).

Estimation of total protein:

Total protein was determined by the Lowry's method using bovine serum albumin as a standard (Lowry et al., 1951).

13C-NMR spectroscopic analysis of insoluble dextran:

Nuclear magnetic resonance (13C-NMR) spectra of dextran was performed with Spectrometer JEOL JNM-ECA 500 MHz NMR of the Central Laboratory, Faculty of Science, Alexandria University. The insoluble dextran was vacuum dried and then exchanged with deuterium by successive lyophilization steps in D_2O (30 mg were dissolved in 0.4 mL of D_2O for 13C NMR). The chemical shift values were reported in ppm (δ). Various signals were assigned as described by Seymour et al., (1976).

Thin Layer Chromatography:

Paper chromatography was performed to determine the hydrolysis fermentation products of dextran produced by the selected strain (Tanaka et al., 1978). Descending technique was adopted using Whatman no.1 paper and the solvent mixture, n-butanol: acetone: water (4: 5: 1, by volume). Hydrolysis was done with 0.1 normal HCl in boiling water bath for 1 hour. The chromatograms were sprayed with aniline phthalate reagent (dissolving 1.66 g phthalic acid + 0.91 aniline in 48 mL butanol and 48 mL diethyl ether and H_2O).

3. Results

Identification of the Organism:

The isolated strain was Gram-positive, Facultative anaerobic and non-motile rods. The biochemical tests showed that culture was catalase and oxidase negative. It can utilize glucose, maltose, sucrose, lactose, manitol and arabinose but not sorbitol, raffinose, xylose and melibiose. The strain was confirmed as a member of genus *Lactobacillus acidophilus* ST76480.01 by 16S rRNA gene analysis.The phylogenetic relationship between the gene and some other strains of *lactobacilli* was analyzed to find the molecular relationship as shown in (Figure 1). It was concluded that our isolate of *Lactobacillus* with the accession number of ST76480.1 and the strain of *Lactobacillus* with the accession number of TH9842.1 were having the same origin and were closely related together than with NC 765214.1, NC 0153456.1 and NC 015214.1.

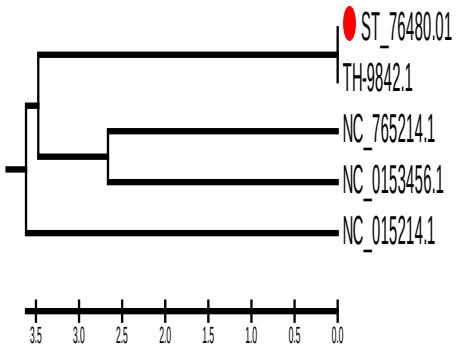

Figure 1. Comparative phylogenetic analysis of *Lactobacillus acidophilus* ST76480.01 with other identified *Lactobacilli*.

Time course for the production of dextransucrase and dextran:

Dextran of high molecular weight was being produced by a bacterium *Lactobacillus acidophilus* ST76480.01. Dextran production and enzyme activity by *Lactobacillus acidophilus* ST76480.01 with reference to time (4-96 hours) were shown in (Figure 2). Maximum enzyme activity was observed at 48 hours of incubation, it was 4.24 DSU/mL/hr. The optical density at 600 nm, increased from 0.055 g/L up to 1.515 g/L at 48 hours and then entered the decline phase. The enzyme activity correlated well with the bacterial growth of *Lactobacillus acidophilus* ST76480.01. The highest production of dextran (2.9 mg/mL) with an increasing in viscosity by (1.62%) were also obtained after 48 hours of incubation.

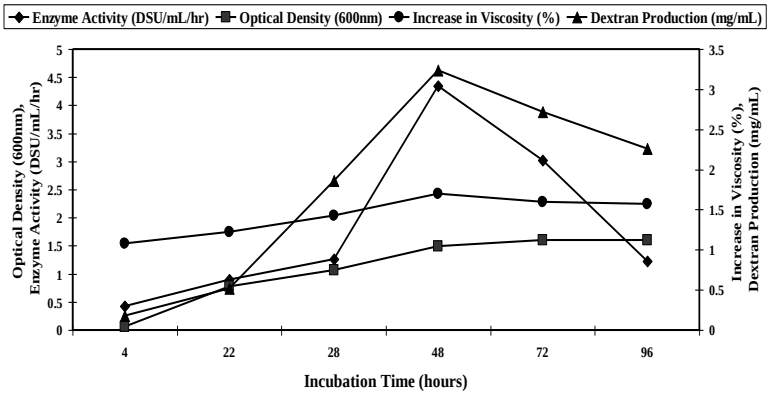

Figure 2. Time course for dextran and dextransucrase production by *Lactobacillus acidophilus* ST76480.01. 15 % sucrose medium.

Optimization of medium components for dextran production and dextransucrase activity:
Selection of the most suitable carbon source:

Sucrose of the broth cultivation medium was substituted with glucose, fructose, maltose, lactose, molasses and starch as 10% (wt/vol) each at a time. As shown in (Figure 3), sucrose and molasses were the best for dextran production (3.04, 3.60 mg/mL), respectively and dextransucrase activity production (4.27, 4.30 DSU/mL/hr) respectively.

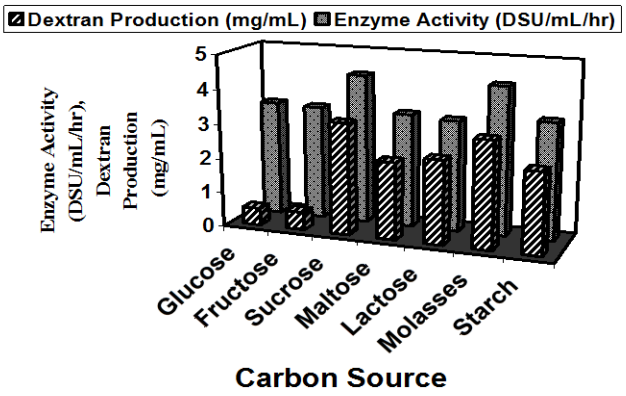

Figure 3. Effect of different carbon sources on dextran and dextransucrase production by *Lactobacillus acidophilus* ST76480.01.

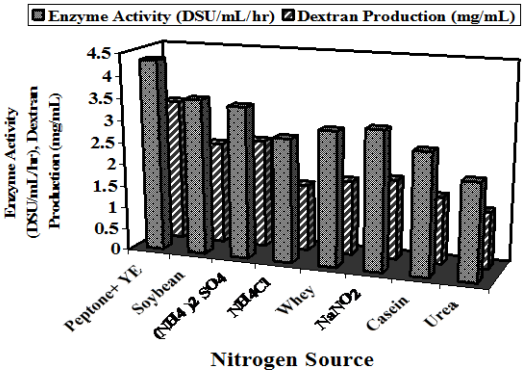

Figure 4. Effect of different nitrogen sources on dextran and dextransucrase activity production by *Lactobacillus acidophilus* ST76480.01.

Selection of the most suitable nitrogen source:

Yeast extract and peptone of the broth medium were substituted with soybean, $(NH4)_2SO_4$, NH_4Cl, whey, $NaNO_2$, casein and urea each at a time as 1% (wt/vol) in equivalent nitrogen basis. Data in (Figure 4) indicated that yeast extract and peptone together were favored as nitrogen sources producing (3.2 mg/mL) of dextran polymer and (3.51 DSU/mL/hr) dextransucrase activity.

Effect of pH and temperature on dextran and dextransucrase production:

Dextran production by *Lactobacillus acidophilus* ST76480.01 was also observed between pH 2 to 14 when incubated at 25°C for 48 hours. At initial pH 8.3, maximum dextran and dextransucrase production (3.44 mg/mL and 4.65 DSU/mL/hr) respectively, were achieved. Dextran production was determined at different temperatures from 15°C to 45°C. The maximum dextran production (3.30 mg/mL) and the greatest enzyme activity (4.62 DSU/mL/hr) by *Lactobacillus acidophilus* ST76480.01 were achieved at 30°C (Data not shown).

Selection of the influential media components for process modeling:

The medium nutrient components were screened by applying the Plackett-Burman matrix. Eight combinations, dextran production and dextransucrase activity were recorded in (Tables 3 and 4).

Table 3 showed that, trial 5 followed by trial 1 yielded the highest amount of dextran polymer (0.282, 0.262 mg/mL) respectively. The degree of significance of sucrose concentration was the highest (95%). The increase in sucrose concentration resulted in an evaluation in the production of dextran polymer. Table4 indicated that, trial 1 followed by trial 3 yielded the highest dextransucrase activity (2.450, 2.415 DSU/mL/hr) respectively. Also, these results recorded that the degree of significance of K_2HPO_4 concentration was the highest (95%). The decrease in its concentration resulted in an increase in the enzyme activity. It was deduced from (Figure 5) that sucrose was the most significant variable for dextran production and K_2HPO_4 was the most significant variable for dextransucrase activity.

Table 3: Degree of positive and negative effects of independent variables on the production of dextran polymer by *Lactobacillus acidophilus* ST76480.01 according to levels in the Plackett–Burman experiments

Variable	Sucrose		Peptone		YE		K_2HPO_4		$MnCl_2$		NaCl		$CaCl_2$	
	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	0.26	0.03	0.26	0.16	0.26	0.17	0.17	0.26	0.26	0.17	0.16	0.26	0.17	0.26
	0.17	0.19	0.17	0.28	0.16	0.03	0.28	0.16	0.03	0.16	0.03	0.17	0.16	0.28
	0.16	0.09	0.03	0.19	0.19	0.28	0.19	0.03	0.28	0.09	0.28	0.19	0.03	0.09
	0.28	0.16	0.09	0.16	0.09	0.16	0.09	0.16	0.19	0.16	0.09	0.16	0.19	0.16
Mean	0.22	0.12	0.14	0.20	0.18	0.16	0.18	0.15	0.19	0.15	0.14	0.19	0.15	0.20
Main effect	0.102		-0.057		0.018		0.031		0.04		0.055		-0.047	
T-value	2.225		-0.993		0.293		0.503		0.669		-0.943		-0.944	
Deg.of sign.	95 %		95 %		95 %		95 %		95 %		95 %		95 %	

$t_{\alpha 95}$= 1.943 $t_{\alpha 90}$= 1.439. Deg. of sign. : Degree of significance

Table 4: Degree of positive and negative effects of independent variables on the production of dextransucrase enzyme activity by *Lactobacillus acidophilus* ST76480.01 according to levels in the Plackett–Burman experiments

Variable	Sucrose		Peptone		YE		K_2HPO_4		$MnCl_2$		NaCl		$CaCl_2$	
	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	2.45	2.39	2.45	2.41	2.45	2.38	2.38	2.45	2.45	2.38	2.45	2.45	2.38	2.45
	2.38	2.36	2.38	2.37	2.41	2.39	2.37	2.41	2.39	2.41	2.39	2.38	2.41	2.37
	2.41	2.35	2.39	2.36	2.36	2.37	2.36	2.39	2.37	2.35	2.37	2.36	2.39	2.35
	2.37	2.41	2.35	2.41	2.35	2.41	2.35	2.41	2.36	2.41	2.35	2.41	2.36	2.41
Mean	2.40	2.38	2.39	2.39	2.39	2.39	2.37	2.41	2.39	2.39	2.38	2.40	2.39	2.39
Main effect	0.023		0.006		0.006		-0.047		0.006		-0.017		-0.006	
T- value	0.107		0.264		0.242		-3.602		0.242		-0.783		-0.287	
Deg.of sign.	95 %		95 %		95 %		95 %		95 %		95 %		95 %	

$t_{\alpha 95}$= 1.943 $t_{\alpha 90}$= 1.439. Deg. of sign. : Degree of significance

Maximum dextran yield (4.24 mg/mL) and the highest dextransucrase activity (4.64 DSU/mL/hr) were obtained when 15% sucrose concentration was used in the fermentation medium but there was a decrease in percent conversion of sucrose to dextran, which ultimately affected the yield (Data not shown).

By decreasing the concentration of K_2HPO_4 from (25 g/L) to (15 g/L), the dextran production reached its maximum value (4.34 mg/mL) but the highest dextransucrase (4.45 DSU/mL/hr) was obtained at (10 g/L) of K_2HPO_4 (Data not shown).

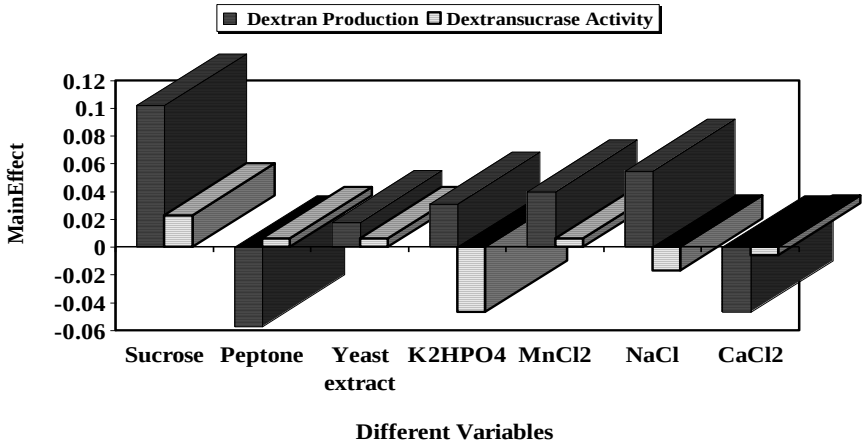

Figure 5. Main effect of dextran production and dextransucrase activity according to Plackett–Burman design.

13C-NMR spectroscopic analysis of insoluble dextran:

The insoluble dextran from *Lactobacillus acidophilus* ST76480.01 showed six prominent 13C NMR resonances at 500 MHz: 104.245, 80.437, 76.192, 75.353, 63.258 and 59.739 ppm (Figure 6), which were the characteristics of linear dextran. A part from these six peaks, which correlated with six signals of linear dextran, the spectra of insoluble dextran also contain minor peaks indicative of branching. There was a weak signal at 98.111, 96.185 and 95.641 ppm, which were downfield of peak at 104.245, meaning that the branching occurred only through 3,6-di-*O*-substituted residues, it showed that the β-anomeric carbon was formed due to the presence of fructansucrase.

The resonance peaks obtained with the insoluble dextran from *Lactobacillus acidophilus* ST76480.01 were compared to dextrans from other strains using the data reported earlier.

Thin Layer Chromatography of dextran:

TLC was carried out using paper chromatography of the hydrolyzed polymer which precipitated from *Lactobacillus acidophilus* ST76480.01 using glucose, fructose and sucrose as controls, results indicated that glucose was the major product of the precipitated materials followed by fructose as a small quantity.

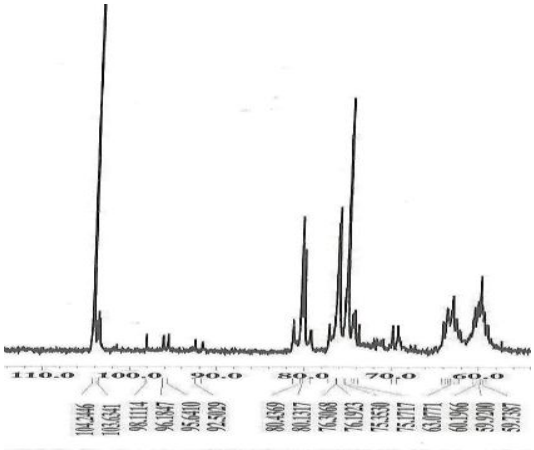

Figure 6. 13C-NMR spectroscopic analysis (500 MHz, D_2O) of insoluble dextran produced by *Lactobacillus acidophilus* ST76480.01.

4. Discussion

The isolated strain was confirmed as a member of genus *Lactobacillus acidophilus* ST76480.01 by 16S rRNA gene analysis (Kullen et al., 2000). Dextran production and enzyme activity by *Lactobacillus acidophilus* ST76480.01 were correlated with time, maximum values of parameters were obtained after 48 hours of incubation. The enzyme activity correlated well with the bacterial growth of *L. mesenteroides* CMG713 (Sarwat et al., 2008**)**. The production of the dextran and the enzyme activity increased with time and after reaching maxima at 20 hours.

Upon substituting sucrose of the broth cultivation medium for *Lactobacillus acidophilus* ST76480.01 with glucose, fructose, maltose, lactose, molasses and starch it was observed that sucrose and molasses were the best for dextran and dextransucrase production. Cortezi et al. (2005) and Santos et al.

(2000) studied the production of dextransucrase and dextran by *Leuconostoc mesenteroides* FT 045 B and *Leuconostoc mesenteroides* NRRL-B512 (f) respectively using sucrose as a carbon source. In another study maltose was used as an acceptor molecule to study its effect on dextran yield and intermediate oligosaccharides were formed that ultimately decreased dextran yield (Rodrigues et al., 2005).

Data in the manuscript indicated that yeast extract and peptone together were favored as nitrogen sources. These results were agreed with many other searchers (Sarwat et al., 2008; Sawale and Lele, 2010) who utilized the same nitrogen source, for production and characterization of a unique dextran from an indigenous *Leuconostoc mesenteroides* CMG713 and dextran production by *Leuconostoc* sp., isolated from Fermented Idli Batter by *Leuconostoc mesenteroides* AB326298, respectively.

At initial pH 8.3 and at 30°C, maximum dextran and enzyme yield by our dextran forming selected strain were achieved. Sarwat et al. (2008) indicated that when the initial pH for dextran production by *L. mesenteroides* CMG713 was kept 7.0 before sterilization, maximum dextran production was achieved. Cortezi et al. (2005) showed that the maximum activity was reached when dextransucrase fermentation by *L. mesenteroides* FT045 B in medium containing 3 and 4% of sucrose at 25°C.

The medium nutrient components were screened by applying the Plackett-Burman matrix. It was deduced that sucrose was the most significant variable for dextran production and K_2HPO_4 was the most significant variable for dextransucrase activity. Maximum dextran yield and the highest dextransucrase activity were obtained at 15% sucrose concentration. Sucrose was an essential carbon source for dextransucrase and dextran synthesis by *Leuconostoc* species (Kartikeyan et al., 1996). Perhaps higher concentration of sucrose in the fermentation medium had an inhibitory effect, known as substrate inhibitory effect, which decreased dextran production due to increased viscosity during fermentation which results in mass transfer limitation of nutrients (Kim et al., 2003). By decreasing the concentration of K_2HPO_4, the dextran production reached its maximum value but the highest dextransucrase activity was obtained at (10 g/L) of K_2HPO_4. Sarwat et al. (2008) studied the production and characterization of dextran from *Leuconostoc mesenteroides* and observed that maximum dextran production was obtained at (15 g/L) of K_2HPO_4.

TLC was carried out using paper chromatography of the hydrolyzed polymer indicated that glucose was the major product of the precipitated materials followed by fructose as a small quantity.

The insoluble dextran from *Lactobacillus acidophilus* ST76480.01 showed six prominent 13C NMR resonances at 500 MHz, which were the characteristics of linear dextran. According to Seymour et al. (1979), the resonances of C-2, C-3 and C-4 were displaced downfield into the 90-98 ppm region, which is the resonance region known for branched linkages. The resonance peaks obtained with the insoluble dextran from *Lactobacillus acidophilus* ST76480.01 were compared to dextrans from other strains using the data reported earlier. The six major resonance peaks of the linear dextrans from the strains *Leuconostoc mesenteroides* NRRL B-640, *L. mesenteroides* NRRL B-1399, *L. mesenteroides* NRRL B-1355 and *Streptobacterium dextranicum* B-1254. It has been shown earlier that all dextrans contain six major resonance peaks for linear linkages and additional peaks for branching (Gorin, 1981; Shukla et al., 2011).

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