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Purification and characterization of glucosyltransferases produced by *Streptococcus pneumoniae* P3 and its kinetic properties with some coumarin derivatives

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The glucosyltransferase (E.C.2.4.1.5) from Streptococcus has been partially characterized by many researchers. In this study, we purified glucosyltransferase to homogeneity from *Streptococcus pneumoniae* P3 with about 6.11 purification fold and a 38% recovery by ion-exchange and gel filtration chromatographic. Glucosyltransferase was estimate the molecular weight which was shown to be 63.095 KD by using gel filtration chromatography using Sephacryl S-200 and the optimum temperature for glucosyltransferase enzyme activity was 37 ^{o}C. The optimum pH for enzyme activity 6.5 and the enzyme kept its fully activity at pH 6.2 for one hour. The glucosyltransferase enzyme kept its fully activity at temperature 37^{o}C for one hour. The enzyme was activation energy (Ea) of glucosyltransferase was (4.881 kcal/ mole) and Q10 between 25 to 35 is 1.238. The effects of compounds Coumarin, 7-ethyl-4-methyl coumarin, 7dimethyl-6-nitro coumarin and 7-hydroxy-4-methyl coumarin on enzyme that decrease the activity more than 60%.

Keywords: ***Streptococcus pneumoniae*, glucosyltransferase, coumarin derivatives,**

INTRODUCTION

Glycosyltransferases (GTs) are ubiquitous in nature and are required for the transfer of sugars to a variety of important biomolecules. This essential enzyme family has been a focus of attention from both the perspective of a potential drug target as well as a catalyst for the development of vaccines, biopharmaceuticals and small molecule therapeutics[1].

Wu et al., (2010)[2] Found that the purified glycosyltransferase complexes indeed possess glycosyltransferase activity *in vitro*. The reaction conditions (pH, temperature, and metal ions) for the enzyme complex were optimized and would be useful in characterizing other Gtf homologues.

Glycosyltransferases (GTs) are a large, diverse family of enzymes that catalyze the transfer of activated sugars, also termed donor substrates, to acceptor substrates that include a vast range of biomolecules such as a proteins, lipids, nucleic acids or other glycoconjugates. Donor substrates are typically activated mono- or oligosaccharides in the form of nucleotide sugars, such as uridine diphosphate glucose (UDP-Glc), guanosine diphosphate mannose (GDP-Man), or cytidine monophosphate N-acetylneuraminic acid (CMP-NeuAc), but can also include dolichol-phosphate linked to mono- or oligosaccharides or

other lipid linked donor sugars.[3] Therefore, the aim of the study were to isolate and identify a local glucotransferases producing strain of *Streptococcus pneumoniae* P3, and to purify and characterize the glucotransferases produced by the bacterium.

MATERIALS AND METHODS

One hundred twenty sample were isolate from throat swap, saliva , and sputum that taken from hospital for children protection, central Teaching Hospital of peadriatic, and teaching laboratory of Medical city. Cultured on blood agar at 37 ^{o}C in candle jar for 24 hr. It was proved that these bacteria were of the same species of the *streptococcus based* on the phenotypic characteristics of the colonies. Pairs and chains with a cell diameter of 0.5 – 1.25 µm **.**

Enzyme activity had estimated according to the modification method described by Al-Hebshi *et al* (2005) The reaction mixture (total volume 500 µl) consisted of 300 µl sodium acetate buffer 0.1M, pH 6.2 supplemented with 5% sucrose, 150 µl of the crude GTFs extract, and 50 µl potassium phosphate buffer (control). The mixture was incubated at 37^{o}Cfor 1 hrs. adding 1 ml 100% ethanol followed by centrifugation as above. The glucan pellet was hydrolyzed by boiling in 300–400 µl of 1 M HCl for 30 min and then neutralized by addition of an equal amount of 1 M NaOH. Finally, glucans were quantified using the phenol-sulphuric acid method Dubois et al., (1956) . Protein concentration was assayed by the method of Bradford (1976) and Stoscheck (1990)[7], with bovine serum albumin as standard.

Isolation of Streptococcus pneumoniae

The Bacterial isolate was grown on the surface of blood agar medium and incubated anaerobically at 37^{o}C for 24 hrs to screen by the production of a-hemolytic colonies on blood agar and by optochin sensitivity to confirm identity as *S. pneumoniae*. Bacteria were grown to mid-log phase in Todd–Hewitt broth containing 0.5% yeast extract anaerobically and stored in 10% glycerol at -80◦C.(Onwubiko et al.,2007)

Ion exchange chromatography

The exchanger DEAE-cellulose was prepared and packed into column following the method described by Schutte et al., (1979). A column with a diameter of (7.5 x 3.5 cm) was equilibration with 0.05 M phosphate buffer pH 7.5. Partially purified concentrated GTFs (12 ml) were separately passed after loaded onto the column carefully. Then (100 ml) of (0.05M) phosphate buffer pH (7.5) was added. Proteins were eluted by using (200 ml) of a stepwise salt concentration from (0.05-0.3 M) in 0.05M phosphate buffer (pH 7.5). Fractions of (5 ml) were collected and absorbency was monitored at (280 nm). The presence of the GTFs were estimated from each fraction of the major peaks then protein concentration and specific activities were determined for the collected active fractions.

Gel filtration chromatography

Sephacryl S-200 column (67x2.1cm) was prepared and packed according to the instruction of the manufacturing company. The column was equilibrated with (0.3 M) phosphate buffer (pH 7.5) at a flow rate of (50 ml/hour). A (3 ml) sample of each concentrated partially purified GTFs was added to the column, carefully using pasture pipette. Elutions of proteins were done with the application of (200 ml) of (0.3 M) phosphate buffer (pH 7.5). A (5 ml) fraction were collected for each GTF then protein contents were estimated by measuring the absorbency at (280 nm), The major peaks for each GTF were determined by plotting the absorbency of protein fractions versus the elution volumes. GTF activity was determined for each fraction of the major peaks. Protein concentrations and specific activities were also determined for the collected fractions of the major peaks of the different GTF.

Characterization of GTF

Determination of optimum pH for GTF

Buffers of different pH were prepared according to Chamber and Richwood(1993).

A-Indirect method: (measurement of optimal pH for enzyme stability). One hundred microliter of the purified enzyme solution was mixed with 100 µl buffer of different pH, from 4 to 9.5 (each in a single test tube) separately. Tubes were incubated for 30 min. in a water bath at 37^{o} C. Enzyme activity was measured for all the solutions simultaneously.

B-Direct method: (measurement of optimal pH for enzyme activity). Tubes which contained mixtures of enzyme solution and buffers of different pH were prepared as in the indirect method. Enzyme activity was measured directly for all of the solutions in a sequenced manner without incubation.

Determination of optimum temperature for GTF stability and activity:

Determination of optimal temperature for enzyme stability: one hundred μl of enzyme solution was incubated for 30min. with 100 μl of phosphate buffer at different degree of temperature ranging from 20 °C to 60 °C in 5 degree increments. After the end of the incubation periods, enzyme activity was measured for all of the tubes simultaneously.

Determination of optimal temperature for enzyme activity: Enzyme activity was measured, with one modification, which is the change in incubation temperature of the enzyme and substrate from 20 °C to 60° C in 5 degree increments.

Determination of the molecular weight

Molecular weight was determined by gel filtration chromatography,(Laue and Rhodes, 1990)[11] and the standard proteins used for the standard curve and their relevant molecular weights were (Catalase, 230000; Arginine deaminase , 125000; Alkaline phosphatase, 80000; Bovine serum albumin, 67000; Pepsin 34000).

Solution for the measurement of GTF Kinetic constants

It was prepared as described previously in (It was prepared by dissolving (50g) of sucrose in (100 ml) of distilled water and sterilized by filtration.). From this stock solution the concentrations (0.01, 0.02, 0.025, 0.04, 0.05, 0.075, 0.1, 0.125, 0.15, 0.2 and 0.25mM) were prepared by diluting with (0.3M) phosphate buffer pH (7.5). The kinetic values Km and Vmax were determined using Lineweaver-Burk double reciprocal plot.

RESULTS

Streptococcus pneumoniae P3 was grown on chocolate agar overnight at 37 C in candle jar. Later collecting the growth by sterilize swab and washed by normal saline ,then lysis , breaking the cell wall of bacteria with 0.2% sodium deoxycholate, 0.02% sodium dodecyl sulfate, 0.1% sodium azide, 0.3 M sodium citrate; pH 7.8 after that estimate the enzyme activity in crude supernatant. The Enzyme activity 119.380 (U/ml) ,and specific activity 2540 (U/mg).

Ionic Exchange Chromatography

Glucosyltransferase enzyme was obtained by using buffer solution at concentration of 20mM Tris –base pH 8.3. Absorbance of eluted fractions were measured at 280 nm upon the arrival of absorbance to the line of zero (line base), then same buffer with stepwise of NaCl (0.125-1M) used to elute the bounded protein. Ionic exchange chromatography patterns showed two protein peak in wash elution and two peaks in stepwise elution. Only one peak among the stepwise elution peaks represented enzyme activity (tubes 12-19). Those fractions pooled and tested for specific activity (9343.731U/mg) a fold purification of (3.67) and enzymic yield of (62.92%) in parts. (Figure 1).

Gel filtration chromatography

Purification carried out by a gel filtration using Sephacryl S-200. Enzymes fraction from DEAE cellulose were pooled and passed through gel filtration column. The fractionation yielded two protein peaks as absorbance reading at 280nm (wave length), only one peak where appeared when reading absorbance at wavelength of 280 nm and when determined for enzyme activity in resulting parts enzyme activity recorded in (16-17), the specific activity reached (15541.793U/mg), fold of (6.11) and a yield (38.5%) as mentioned in table (1) and figure (2).

The molecular weight was estimated by gel filtration depending on the size of the separated molecules with their charge .It was possible that the different methods of estimation may be used.[13]. Sephacryl S-200 (1.5 x87) cm was used for estimation the molecular weight of purified glycosyltransferase from *streptococcus pneumoniae* P3 illustrated in figure (3). According to the logarithm molecular weight and elution volume/void volume (Ve/Vo) Table (2) standard curve the calculated (MW) of the glycosyltransferase found to be (63.095) KD.Gel granules working as a molecular sieve. Small molecules intervene in gel granules with slow movement, while large molecules with high molecular weights move between gel grains and comes out of column faster than the first proteins in this way. The glycosyltransferase enzyme was purified 6.11 fold with 38.5 % recovery by gel filtration chromatography, DEAE- Cellulose ion-exchange chromatography and gel filtration, as summarized in (Table 1).

The optimum pH for enzyme activity

Figure (4) shows the effect of reaction solution pH on the glycosyltransferase activity. It has been noticed that the optimum pH was 6.5 for glycosyltransferase activity and the activity began to decrease between pH values of 7-7.5.

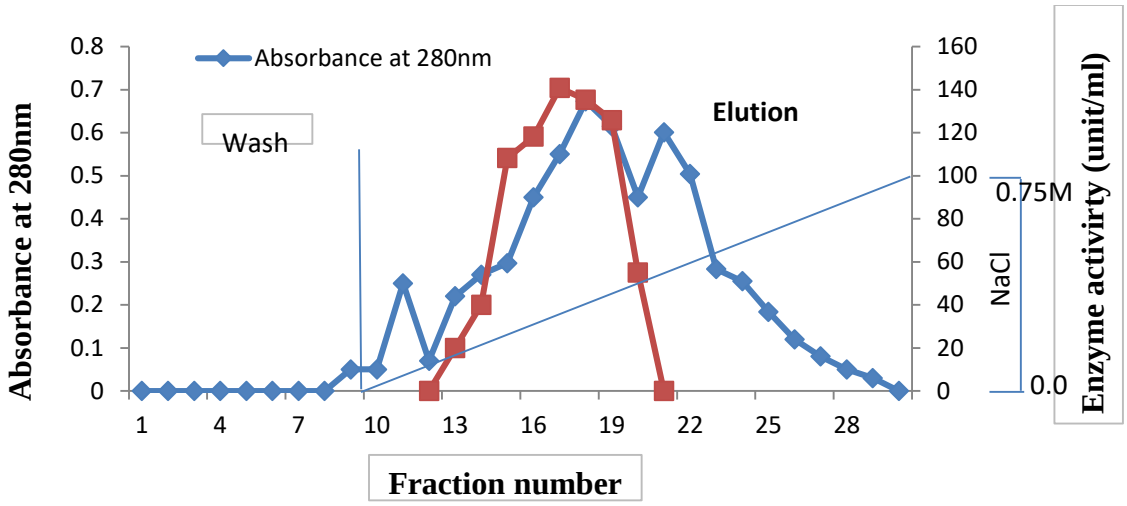

Figure (1) : The ion -exchange chromatography of glycodyltransferase enzyme from *Streptococcus pneumoniae* P3 through DEAE Cellulose column (2x8) cm . The column was calibrated with 20mM Tris-base pH 8.4, flow rate 60 ml/hrs and 5ml fraction.

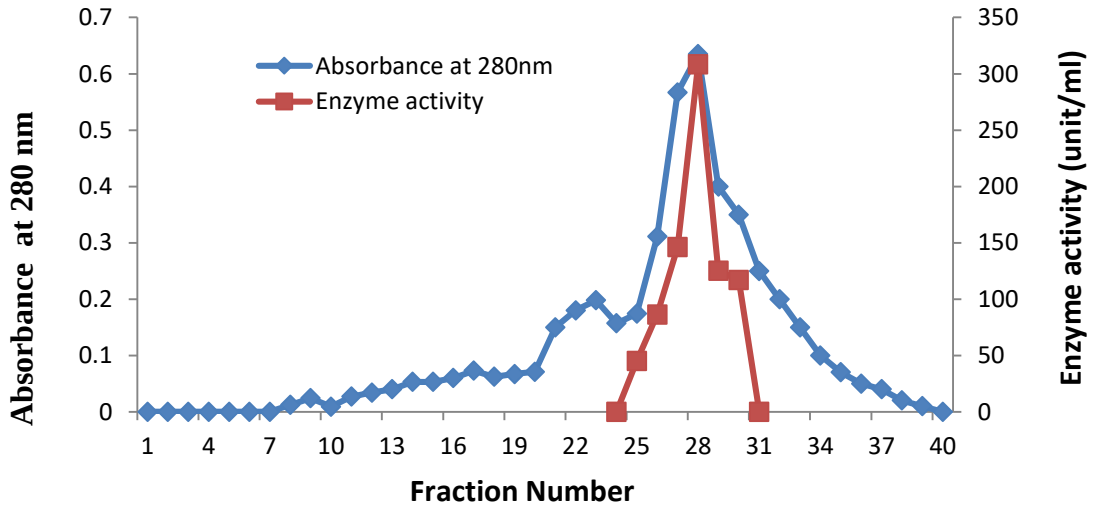

Figure (2):Gel filtration chromatography for purified glycosyltransferase from *S.pneumoniae* P3 by using Sephacryl S-200 column (1.5x87) cm. The column was calibrated with 20mM Tris-base pH 8.3, flow rate 60 ml/hrs and 5 ml/fraction.

Table(1):Purification steps of glycosyltransferase produced from *Streptococcus pneumoniae* P3 .

Steps	Volume (ml)	Enzyme activity (U/ml)	Total activity (Units)	Protein (mg/ml)	Specific activity (U/mg)	Fold	Yield (%)
Crud extract	50	119.380	5969	0.047	2540	1	100
DEAE-cellulose Ionic- exchange	20	187.809	3756.18	0.0201	9343.731	3.67	62.92
Gel-filtration Sephacryl- S200	15	225.356	3380.347	0.0145	15541.793	6.11	38.5

The optimum pH for enzyme stability

Figure (4) illustrates the pH profile for enzyme stability, the purified enzyme incubated at different pH values ranged from (5-7.5)for one hour at 37°C. The obtained results showed a maximum stability at pH 6.2 as the enzyme retained its entire activity. The enzyme was stable at pH 6 and retained more than 80% its entire activity. while at pH values 5,5.5,6,6.5,7, and 7.5 the enzyme lost about 42-77% of its total activity.

The optimum temperature for enzyme activity

Each enzyme works within a range of temperature specific to the type organism. Purified glucosyltransferase from *S.pneumoniae* P3 showed the highest activity at 37°C figure (5).The activity at temperature 25,30,35 and 40 was higher than, 45 and 50 °C. Wu et al.,(2010)[2] clarified The optimal temperature for the complex is 37°C . The presence or absence of divalent ions did not alter the activity.

The optimum temperature for enzyme stability

Figure (6) explains the influence of temperature on purified glucosyltransferase stability upon one hour incubation at different temperature ranged from 25-50°C. The enzyme showed a maximum stability at 37**°**C as it retained the entire activity, besides the enzyme retained move than 60% of its entire activity at temperatures between 30-35**°**C .

The effects of different concentration of sucrose on pure enzyme.

Figure (7) shown that the activity of enzyme increased with increasing of substrate concentration that ranging (2,4,5,6,8 and 10%) the activity was 77.952,85.095 ,106.523 ,148.428, 174.142 and 176.047) U/ml respectively. That is means the substrate saturated the binding site that resulting stable the activity in concentration 8 and 10 %. It can be noticed that enhancing substrate concentration enhances the rate of the reaction because of increasing the collisions between substrate and enzyme molecules until reaching a certain concentration, after that further increasing in substrate concentration has no effect on the reaction rate because that enzyme becomes saturated and its active site is occupied with substrate concentration become without effect on the reaction rate.

Activation energy (Ea) of glucosyltransferase purified that conversion of substrate to product is calculated according to the relation between the logarithms of observed reaction rate constant and reciprocal of Kelvin temperature (1/T°). From Figure (8) it can be noticed that the activation energy (Ea) of glucosyltransferase was (4.881 kcal/ mole) and Q10 between 25 to 35 is 1.238 .

The effects of inhibitors on purified enzyme :

The enzyme after purified by gel filtration estimate the effects of inhibitors with different concentration on the enzyme activity ,Table (2) illustrate these more effects of compounds Coumarin, 7-ethyl-4-methyl coumarin,7dimethyl-6-nitro coumarin and 7-hydroxy-4-methyl coumarin on enzyme that decrease the activity more than 60%. The substituted that adding to coumarin were increase the inhibition ability of these compounds the oxygen ,methyl ,ethyl and hydroxyl groups these increase the connecting with binding site.

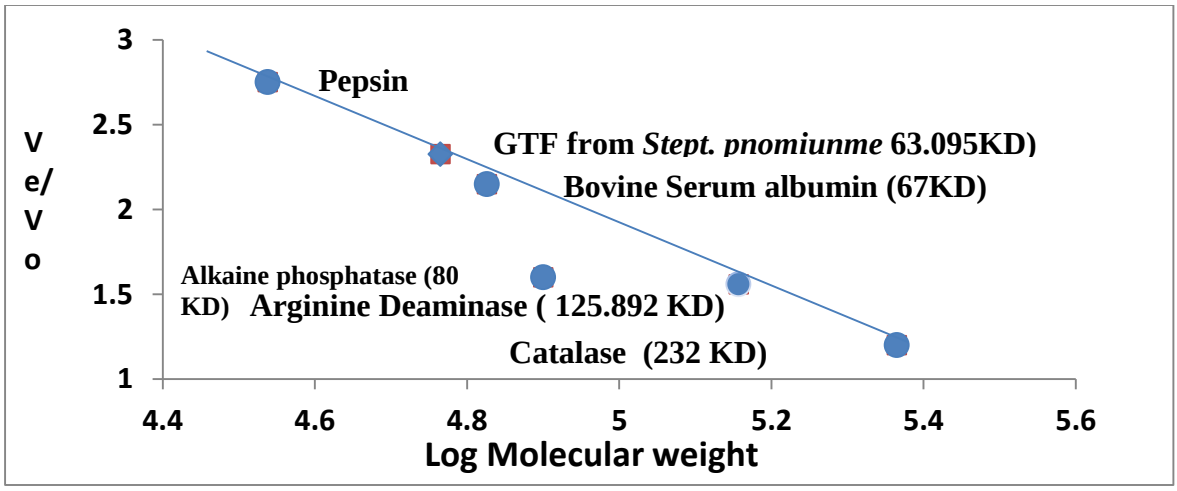

Figure (3): Standard curve to estimate molecular weight of glycosyltransferase enzyme produced by *streptococcus pneumoniae* P3 using gel filtration by using sephacryl S-200.

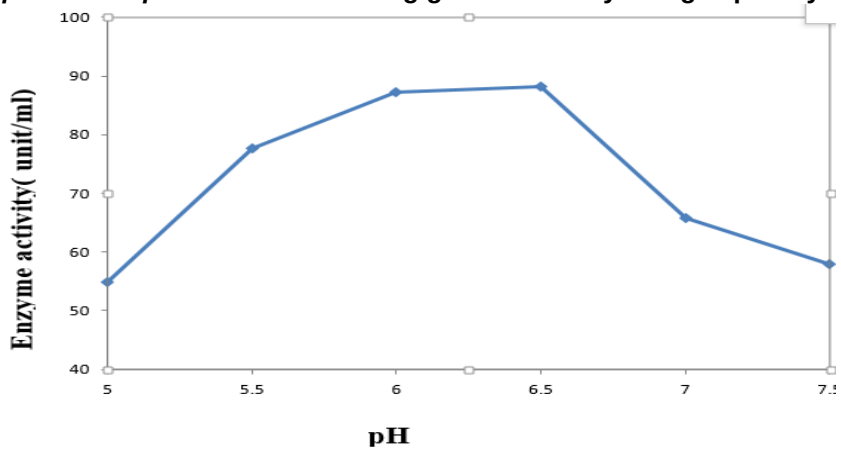

Figure (4): Effect of different pH on activity of purified glycosyltransferase from *streptococcus pneumoniae* P3.

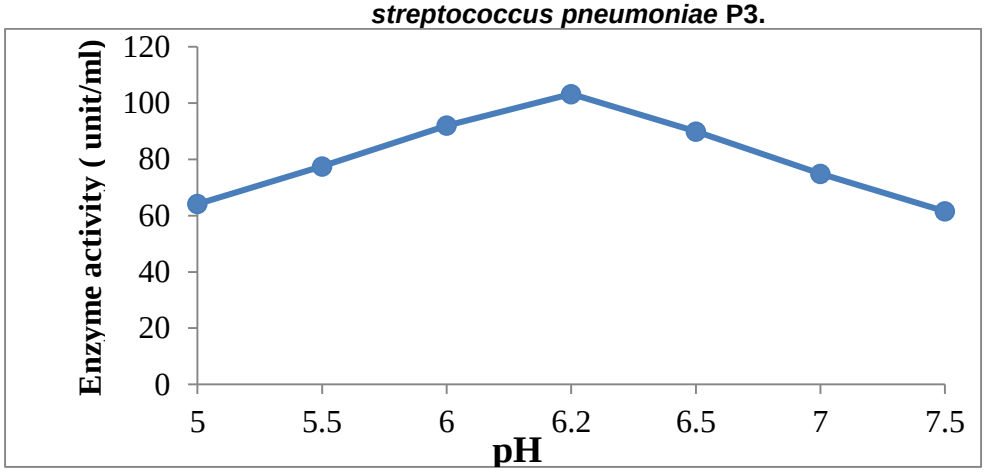

Figure (5): Effect of pH on stability of purified glucosyltransferase f*S.pneumoniae P3.* The purified enzyme incubated at different pH values 5-7.5 for 1 hours at 37 °C.

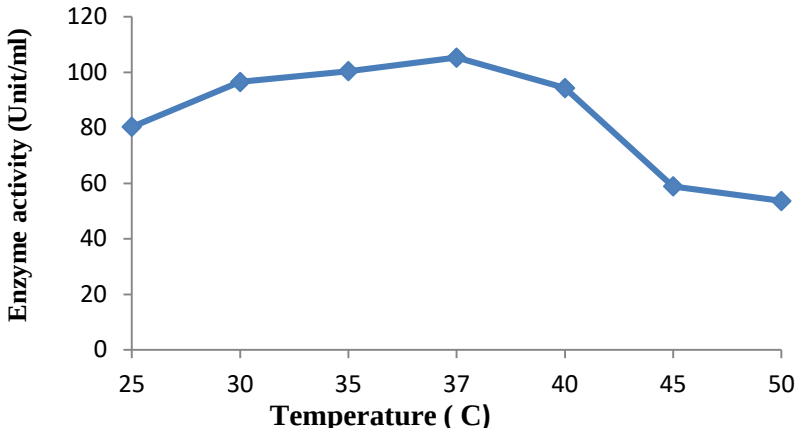

Figure (6): Effect of temperatures on activity of purified glucosyltransferase from *S.pneumoniae* P3.

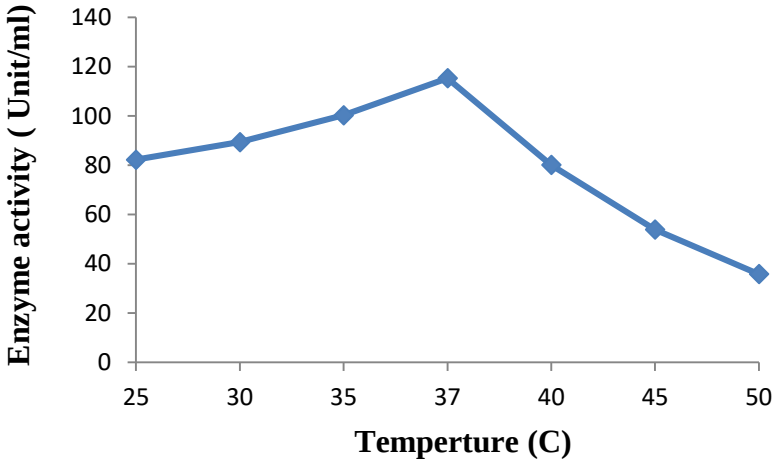

Figure (7): Effect of different temperatures on activity of purified glucosyltransferase enzyme from *S.pneumoniae*, when incubated for one hour at temperature rang form 25-50 ºC.

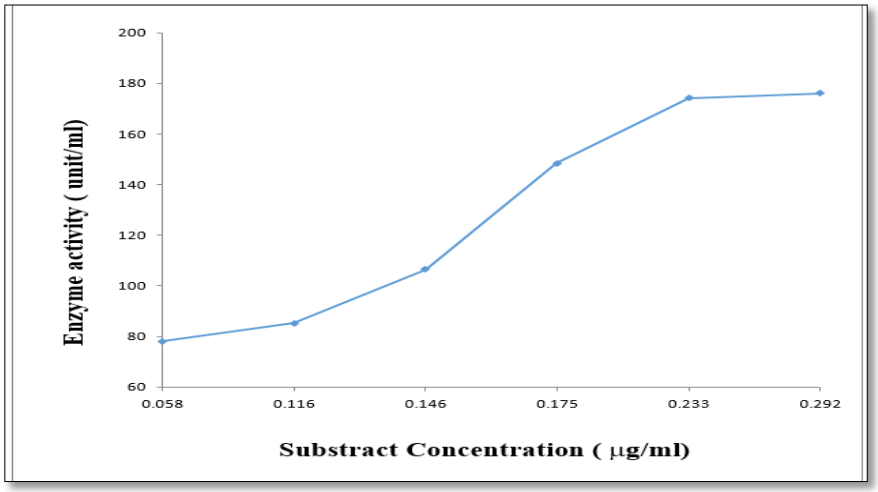

Figure (8): Effects of different concentration of sucrose on glucosyltransferase activity.

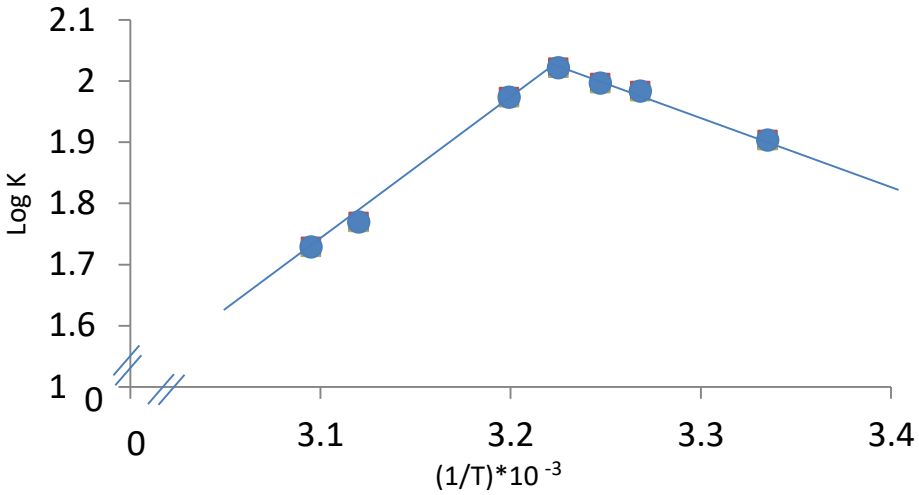

Figure (9): The Arrhenius curve to determined activation energy of glycosyltransferase from *Streptococcus pneumoniae* P4.

	Compounds	Enzyme activity After incubation with 100 µg/ml	Enzyme activity After incubation with 200 µg/ml	Enzyme activity After incubation with 300 µg/ml	Enzyme activity After incubation with 400 µg/ml
1	Coumarin	20.238	10.809	7.238	6.483
2	7-ethyl-4-methyl coumarin	14.33	9.142	8.904	7.774
3	4,7dimethyl-6-nitro coumarin	12.048	9.857	8.587	5.571
4	7-hydroxy-4-methyl coumarin	20.516	12.238	8.428	5.33

DISCUSSION

First, the most useful methods for protein purification depending on the surface molecule charge, the protein and the buffer conditions and the protein will have net a positive or negative charge (Segel,1976). Al-Mudallal et al.,(2010) who mentioned Mutans streptococci (serotype G) GTF has specific activity (0.752 U/mg protein) . The results for molecular weight of glucosyltransferase were agree with another studies by Wu et al.,(2010) estimate the molecular weight in *Streptococcus parasanguinis* was ranging between 45 kDa to 51 kDa. But another study by Wang et al.,(2001) shows the molecular weight of crude glycosyltransferase In *staphylococcus aureus* when genetically engineered in *E. coli* the was~ 32kDa that estimate by SDS-PAGE. While, AL-Jumaily et al.,(2014) mentioned the enzyme activity was 65.7(U/ml) that produced from *Streptococcus mutans*. But Gawande and Patkar (2001) found that the molecular weight of pure α-cyclodextrin glycosyltransferase molecular in *Klebsiella pneumoniae* AS-22 of the pure protein was estimated to be 75 kDa with SDS-PAGE and gel filtration.

The influence of pH effect on the enzymatic protein structure or irreversible denaturation may occur in a high acidic or basic solution which leads to change in the active site of the enzyme so loss of activity is observed. (Whitaker, 1972). The gradual loss in enzyme activity could be because of temperature effect on the tertiary structure of the enzyme and distortion in the active site of protein due to loss of activity to the breakdown of substrates. Wenk and Fernandis, (2007). Wang et al.,(2001) found the optimum pH In *E coli* ranging between 7.5 to 8. The opinion of Wu et al., (2010) glucosyltransferase was a complex Gtf1/2 that more active under acidic conditions (pH 5 to 7) in *Streptococcus parasanguinis*. The value is within physiological activation energy range stated by Whitaker and Bernhard (1972) which is located between (1-25 kcal/mol) for physiological processes in living organisms.

Al-Majedy et al.,(2017) synthesized 3- amino-(N-aryl substituted)-6-bromo-2H1-benzopyran-2-ones and 6-bromo-3-phenoxy substituted-2H-1-benzopyran-2-ones. All the title compounds were

screened for *in vitro* antitubercular activity against highly virulent. acyl coumarins, 4-hydroxy, and 7-hydroxycoumarins and coumaric amide dimers and were tested against stains of *Bacillus subtilis*, *Staphylococcus aureus* , *Escherichia coli*, and *Pseudomonas aeruginosa*.

The inhibitors activity on *Streptococcus pneumoniae* P4 results are agree with Lee et al.,(2014) which they shown that the low activity associated with hydroxylated coumarins against *E. coli* with respect to biofilm formation hydroxylation at position 4 or position 8 .while the same modification at position 7 led to enhanced activity. Di-hydroxylation of coumarin at positions 6 and 7 led to a reduction in activity compared to coumarin compound.

Olender et al.,(2018) In biological systems, the nitro group undergoes enzymatic reduction, which can take place by both a one- or two-electron mechanism. Sequential two-electron reduction of the NO_2 group gives amines via nitroso and hydroxylamine intermediates. The nitro aromatics and amines remains unchanging, but sometimes the nitroso and hydroxylamine intermediates can react with biomolecules to produce compounds having undesired effects. A one-electron reduction of the nitro group produces a nitro radical anion, which is unstable. Under aerobic conditions, it is re-oxidized back to the nitro group by molecular oxygen, which is in turn reduced to form a reactive superoxide anion.

CONCLUSION

We can concluded that the important to purified and characterization of glucosyltransferase from Streptococcus *pneumoniae* to shown how the new inhibitors 7-hydroxy-4-methyl coumarin compound gave high inhibition with 400 μg/ml concentration compared with another inhibitors that may be Possibly due to the structural form which have the hydroxyl groups.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

EFA contributed to the design of the experiments and performed the experimental work. RIAA carried out laboratory tests. EFA wrote the manuscript. All authors read and approved the final version.

REFERENCES

AL-Hebshi, N.N., Nielsen. Ø. and Skaugn *In vitro* effects of crude khat extractson the growth, colonization, and glucosyltransferases of Streptococcus mutans. Acta Odontologica Scandinavica, 63: 136–142.(2005).

Al-Jumaily, E. F.A.; AL-Seubehawy, H.M.Z. and Al-Toraihy, F.A. Isolation and Identification of *Streptococcus mutans* (H5) produced glucosyltransferase and cell-associated glucosyltransferase isolated from dental caries. Int.J.Curr.Microbiol.App.Sci. 3,.6: 850-864.(2014).

Al-Majedy, Y.K., Kadhum, A. A., Al-Amiery ,A. A. and Mohamad A. B. Coumarins: The Antimicrobial agents. Sys Rev Pharm.;8 (1):62-70.(2017).

Al-Mudallal, N.H.A., Al-Jumaily, E.F.A., Muhimen, N.A.A. and Al-Shaibany, A.A. Isolation and Purification of glucosyltransferase from mutans *Streptococcus Sob*rinus (serotype G) local isolate. Iraqi J. of Medical Science. 8 ; 4: 10-18.(2010).

Aram, C. , Shanteri, S., Phillips,G.N. Jr., and Jon, S. T. Glycosyltransferase structural biology and its role in the design of catalysts for glycosylation. Curr. Opin. Biotechnol. 22(6): 800–808.(2011).

Bradford, M. M. A rapid and sensitive method for the quantitation of microorganism's protein utilizing the principleof protein-dye binding. Analytical Bioch.72: 248-254.(1976).

Chamber, J.A.A. and Richwood, D. Biochemistry labfacs: Bios. Scientific Publisher Limited, St Thomas house, Becket Street.

Oxford.(1993).

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A and Smith, F. Colorimetric method for determination of sugars and related substances. Anal Chem. 28:350–356.(1956). Formation and the virulence of Escherichia coli O157:H7. Phyto medicine 21(8–9):1037–

Gawande, B.N. and Patkar, A.Y. Purification and properties of a novel raw starch Degrading-cyclodextrin glycosyltransferase from *Klebsiella pneumoniae* AS-22. Enzyme and Microbial Technology. 28 : 9-10, (7),735-743.(2001).

Lairson, L. L., Henrissat, B., Davies, G. J., and Withers, S. G Glycosyltransferases: structures, functions, and mechanisms. Annu. Rev. Biochem.77,521 555.(2008).

Laue, T.M.and Rodes, D.G. Gel filtration. In: Methods in Ezynmology (ed. Deutscher, M.P.). 182:556-587.Academic Press, Inc.(1990).

Lee J.H., Kim Y.G., Cho H.S., Ryu S.Y., Cho M.H. and Lee J. Coumarins reduce biofilm Formation and the virulence of Escherichia coli O157:H7. Phytomedicine 21(8–9):1037–1042.(2014).

Olender, D.; Żwawiak, J. and Zaprutko ,L. Multidirectional Efficacy of Biologically Active Nitro Compounds Included in Medicines. Journal Pharmaceuticals; 11, 54. 2018).

Onwubiko**,** C., Shires , C., Quin,L. R., Swiatlo , E. and McDaniel , La. S. Characterization of *Streptococcus pneumoniae* Isolated from children with otitis media. Federation of European Microbiological Societies Immunol. Med. Microbiol. 50 ; 119–125.(2007).

Schutte, H., Buchholz, R., Gotz, P., Gotz, P., Schweizer,M. and Grafe, U. Isolation techniques. In: Methods in Biotechnology. Schmauder, H.P.(ed.). J. Internatinal Ltd., Britain.(1997).

Segel, I.H. Biochemical Calculations, 2nd edition, John and Sons. Inc. NewYork.(1976)

Stoscheck, C. M. Quantitation of protein. Methods in Enzymology. 182: 50-69.(1990).Onwubiko, C., Shires , C., Quin,L. R., Swiatlo , E. and McDaniel , La. S. Characterization of *Streptococcus pneumoniae* Isolated from children with otitis media. Federation of European Microbiological Societies Immunol. Med. Microbiol. 50 ; 119–125.(2007).

Wang ,Q. M.; Peery, R. B.; Johnson, R. B.; Alborn,W.E.; Yeh, W. and Skatrud, P. L. Identification and Characterization of a Monofunctional Glycosyltransferase from *Staphylococcus aureus*. J. of Bacteriology. 183, 16: 4779–4785.(2001).

Wenk,M.R and Fernandis,A.Z. A manual for Biochemistry Protocols .Vol.3.editor Jan-Thorston Schantz.British library cataloginig-in-publication data. 2-7.(2007).

Whitaker, J. R. Principles of Enzymology for the Food Science. (ed. Oen, R. F.) Marcel Dekker INC. ; New York. (1972).

Whitaker, J.R. and Bernhard, R.A. Experiment for : An Introduction to enzymology. The Whiber Press. Davis. (1972).

Wu,R., Zhou, M. and Wu, H. Purification and Characterization of an Active N-Acetylglucosaminyltransferase Enzyme Complex from Streptococci. Applied and Environment Microbiology. 76, (24):7966–7971.(2010).