

Phenotypic and Molecular Identification of *Bifidobacterium* sp. Isolated from Infants Feces

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Abstract

One hundred fecal samples from clinically less than one month old infants was collected for isolation of *bifidobacterium* sp. , and these samples was cultured on two different selective media for this bacteria De Mann, Rogosa and Sharpe (MRS) agar and Liver Cysteine Lactose (LCL) agar media . Identification of this bacteria was obtained by morphological characteristic, microscopic feature, biochemical tests, Fructose-6-phosphate phosphoketolase (F6PPK) activity assay and molecular identification by used genus-specific primer . And also detection and amplification of *xfp* gene was achieved by PCR technique and specific primer with a product size 249 bp . Gene expression of *xfp* was measured by detection of F6PPK enzyme in crude extract of bifidobacteria . Different conditions from pH, temperature, incubation period, and culture media was used to optimization of condition for production of F6PPK enzyme from bifidobacteria. The result revealed that pH 7 was optimum pH for growth and production of enzyme while 30 C° was optimum temperature for production of F6PPK enzyme and its reach to maximal production after 18 hr of incubation .

Key words : *Bifidobacterium* , *xfp* gene , fructose-6-phosphate phosphoketolase (F6PPK)

الخلاصة

تم جمع 100 عينة خروج من الاطفال الاصحاء سريرياً والذين لا تتجاوز اعمارهم الشهر لغرض عزل بكتريا *Bifidobacterium* sp. . وتم زرع تلك العينات على نوعين مختلفين من الاوساط الاختيارية لهذه البكتريا De Mann, Rogosa, and Sharpe (MRS) و Lactose liver cysteine (LCL). شخضت بكتريا ال *Bifidobacterium* sp عن طريق الخصائص المظهرية , الصفات المجهرية , الفحوصات الكيموحيوية , فحص فعالية انزيم Fructose-6-phosphate phosphoketolase (F6PPK) بالإضافة الى التشخيص الجزيئي باستخدام البادئ الخاص في تشخيص تلك البكتريا على مستوى الجنس , وايضا تم التحري والتضخيم لموروث ال *xfp* باستخدام تقنية ال PCR والبادئ الخاص في ذلك الموروث مع حجم تضخيم 249 زوج قاعدي . تم قياس التعبير الجيني لموروث ال *xfp* بواسطة التحري عن وجود انزيم ال F6PPK في المستخلص الخام للبكتريا, كما تم استخدام ظروف مختلفة من الاس الهيدروجيني , درجة الحرارة , فترة الحضانة والاوساط الزرعية لمعرفة الظروف المثلى لانتاج انزيم ال F6PPK من تلك البكتريا. اوضحت النتيجة ان الاس الهيدروجيني 7 هو الامثل للنمو وانتاج الانزيم بينما 37° كانت هي المثلى لانتاج الانزيم وقد وصلت الى اعلى انتاجية بعد 18 ساعة من الحضانة .

الكلمات المفتاحية: *Bifidobacterium*, موروث ال *xfp* , انزيم الفوسفوكيتوليز (F6PPK)

Introduction

Bifidobacteriaceae are indigenous components of human and animal gastrointestinal microbiota and are routinely isolated from the human gastrointestinal system, especially the colon; they are the first and most dominant gut inhabitants in early human life. Bifidobacteriaceae are also present in the oral cavity and the vagina (Biavati, et al 2012). Bifidobacterial species are common members of the human gut microbiota , comprising up to 3% of the total fecal adults microflora .they are more numerous in the infant gut , where they form up to 91% of the total microflora in breast-fed babies and up to 75% in formula-fed infants, because of their prevalence in the infant feces suckled at the breast ,bifidobacteria are considered to be beneficial bacteria and they are used in the production of the probiotic products (Woese, 2000).

Bifidobacteria are gram-positive, non-sporeforming, nonmotile, branched, rod-shaped, saccharolytic anaerobic bacteria (also some species can tolerate low amount of oxygen) and traditionally considered as a member of lactic acid bacteria group. It produces acetic and lactic acids from carbohydrates without the generation of CO₂ (Tannock, 2010; Ruiz, *et al* 2012a). Bifidobacteria isolated from human intestines are known to grow at optimal temperature values between 36 and 38°C, but animal strains grow at a higher temperature, approximately 41-43°C. It also grows at optimal pH values ranging from 6.5 and 7.0, however they are inhibited by temperature values under 25 and above 45°C. While *Bifidobacterium thermophilum* that was described as one of the common species in ruminant, this species appears to be practically resistant. It is able to survive or multiply at a relatively high temperature (46°C) and was shown to be highly tolerant to oxygen (Blaser and Falkow, 2009). Members of the genus *bifidobacteria* are fermentative bacteria that utilize a variety of carbohydrates, especially oligosaccharides, resulting in the formation of acetic and lactic acids. Hexose is utilized by unusual metabolic pathways known as the bifid shunt. Bifidobacteria achieve large population sizes in the bowels of humans during the first few months after birth, and its populations are probably enriched in the bowel by some of a huge variety of oligosaccharides present in breast milk. The variety of oligosaccharides in milk may partially dictate the kind of bifidobacteria that establish in the bowel of a given child. Native immune cells harvested from cord blood respond differentially to bifidobacterial species, thus the occurrence of immunological diseases such as childhood allergies may be influenced by the kind of bifidobacteria to which a child is exposed in early life (Tannock, 2010).

Materials and Methods

1- Isolation of bifidobacteria

One hundred stool samples were collected from infants under one month old in Al-Hilla Hospital for Delivery and Infants and Al-Kifil Hospital during the period from November 2013 to March 2014. Sterile screw cap contains normal saline was used to collect specimen to avoid any possible contamination and obtained for suspension, then 50 µl of stool suspension transferred to liver cysteine lactose agar (LCL) and De Mann, Rogosa and Sharpe (MRS) agar supplemented with 0.5 gm/L of L-cysteine-HCL, spreading by sterile loop under sterile condition and incubated at 37°C for 24-48 hr under anaerobic condition.

2-Identification of isolates

First step for identification of bifidobacteria morphologically. Bacterial slides treated with gram stain and examined by microscope under high power lens (1000x), since bifidobacteria appear as gram positive rod or pleomorphic shape (typically V or Y shape). F6PPK activity assay, it's a second step for bifidobacterial identification, the isolates considered to belong to the genus *Bifidobacterium* were diagnostic by the detection of F6PPK activity in cellular extract of bacteria as described by Scardovi and Trovati 1965. Briefly, cells were grown in 10 ml of MRS broth medium at 37°C for 18 hr and harvested by centrifugation 6000 rpm/ min for 10 min, the pellet was washed twice with 5 ml of phosphate buffer supplemented with 0.5 gm/L cysteine. After centrifugation the pellet was suspended in one ml of the same buffer, 50 µl of lysozyme was added and after incubation for one hour, cells were disrupted by adding 0.4 ml hexadecyltrimethylammonium bromide (CTAB 0.45mg/ml) for obtaining crude cell extract, 0.25 ml of reagents (6 mg/ml NaF plus 10 mg/ml sodium iodoacetate and 80mg/ml fructose-6-phosphate) was added to the cells extract. The

reaction was started by incubation 30 min at 37C° and stopped by adding 1.5 ml of hydroxylamine-HCL (13.9%) . After 10 min 1 ml of trichloroacetic acid (15%) and 1 ml of FeCl₃ 6H₂O (5%) were added . A negative control consisting of all reagents except the substrate (fructose-6-phosphate) as well as a control strain used to verify the color . The presence of F6PPK enzyme was revealed by the appearance of red or purple color and measured by spectrophotometer at 435 wave length (Orban and Patterson, 2000).

3-Molecular identification of bifidobacteria and amplification of *xfp* gene

Genomic DNA was prepared according to the procedure of Kate Wilson 1997. Its briefly occur by Incubation approximately 5 ml of liquid culture media with bacteria at optimum condition of growth for 24 hr. transfer 1.5ml of culture to a 1.5 appendorfe tube and spin in microcenterfuge for 2 min at 10000 rpm. the supernatant was discarded and the pellet suspended in 567µl TE buffer and mix by pipetting . Add 30µl of 10% SDS and 3 µl of 20 mg/ml proteinase K to give final concentration of 100 mg / ml proteinase K in 0.5% SDS , mix well and incubate at 37C° for one hr. After this step, the solution should become viscous as the detergent lysis the bacterial cell wall. There should be no needed to predigest the bacterial cell wall with lysozyme . Add 100 µl of 5M NaCl and mix thoroughly ,This step is very important to remove cell wall debris , denatured protein , and polysaccharide . While retaining the nucleic acid in solution . Add 80 µl of CTAB /NaCl solution . Mix thoroughly and incubate 10 min in waterpath 65C°. And then add approximately equal volume (0.7-0.8 ml) of chlorophorm /isomyl alcohol. Mix well and spin at 14000 rpm for 5 min. This step is important to remove the CTAB-protein /polysaccharide complexes . A white interface should be visible after centerfugation . Transfer the aqueous , viscous supernatant to a new microcenterfuge tube and add equal volume of phenol /chlorophorm/isomyl alcohol. Mix well and spin in microcenterfuge 14000 rpm for 5 min., transfer the supernatant to a new appendorf tube and add 0.6 volume of isopropanol to precipitate the nucleic acids. Shake the tube back and forth until a stringy white DNA precipitate become clealy visible . Spin the solution at 14000 rpm for 10 min to precipitate the DNA . Add 500 µl of 70% ethanol and spin at 14000 rpm for 5 min to remove the residual CTAB and then carefully discard the supernatant and briefly dry the pellet and then suspended the pellet in 100 µl TE buffer. Specific primer that used in this study are listed in table (1).

Table (1) genus specific primer and *xfp* gene primer.

Target group	primer	Sequence (5' to 3')	Length (bp)	Product size	Referenc es
<i>Bifidobacterium</i> spp	Pbi F1 Pbi R2	CCGGAATAGCTCC GACCATGCACCACCTGT GAA	13 20	914	Denis and Stephane (2000)
<i>XFP</i> gene	Xfp F Xfp R	GCTCCTACGAGTCCTTC GTG TGGAATCAACCGGGAA GTAG	20 20	249	Gen bank

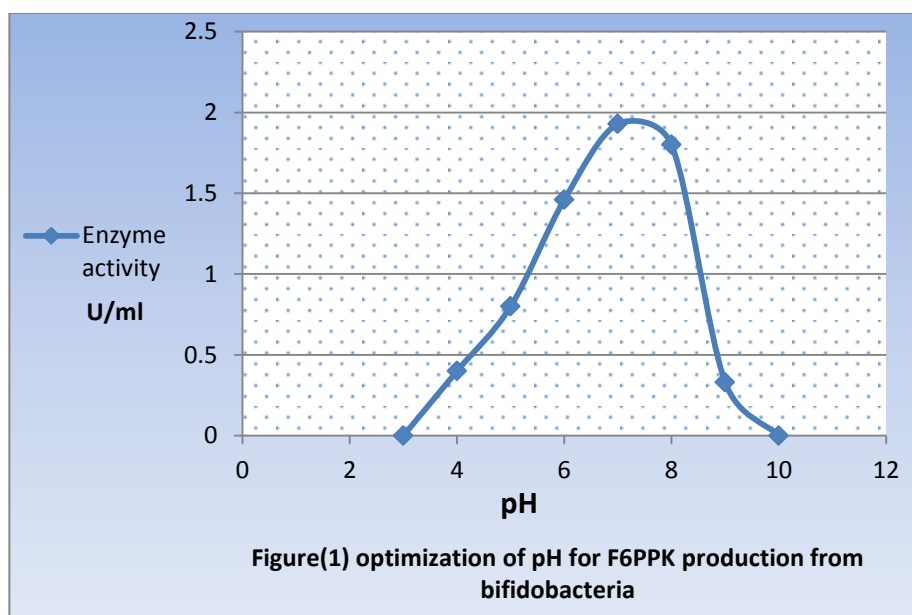
DNA amplification was carried out by using specific primers which explained in table (1) . The amplification reaction was 50 μ l PCR mixture containing : 5 μ l Taq buffer , 5 μ l dNTP , 4 μ l $MgCl_2$, 2 μ l Taq polymerase enzyme , 3 μ l pbi F1 , 3 μ l pbi R2 or (3 μ l xfp F ,3 μ l xfp R) , 3 μ l template DNA (final concentration of DNA was 25 ng) and 25 μ l deionized free water . PCR amplifications were performed with Biometra apparatus with the following conditions : initial denaturation 94C° for 5 min ,followed by 35 cycles of denaturation (94C° for 30 sec) ,annealing (variable temperature 55-60C° for 30 sec) , extension (72C° for one min) and a single final extension step (72C°for 10 min). The amplified fragments were fractionated on 1.5% agarose during one hour at a constant voltage 80V. Genomic fragment were visualized under ultraviolet light followed by digital capturing of the photo by used canon camera. (Denis Roy and Stephane ,2000)

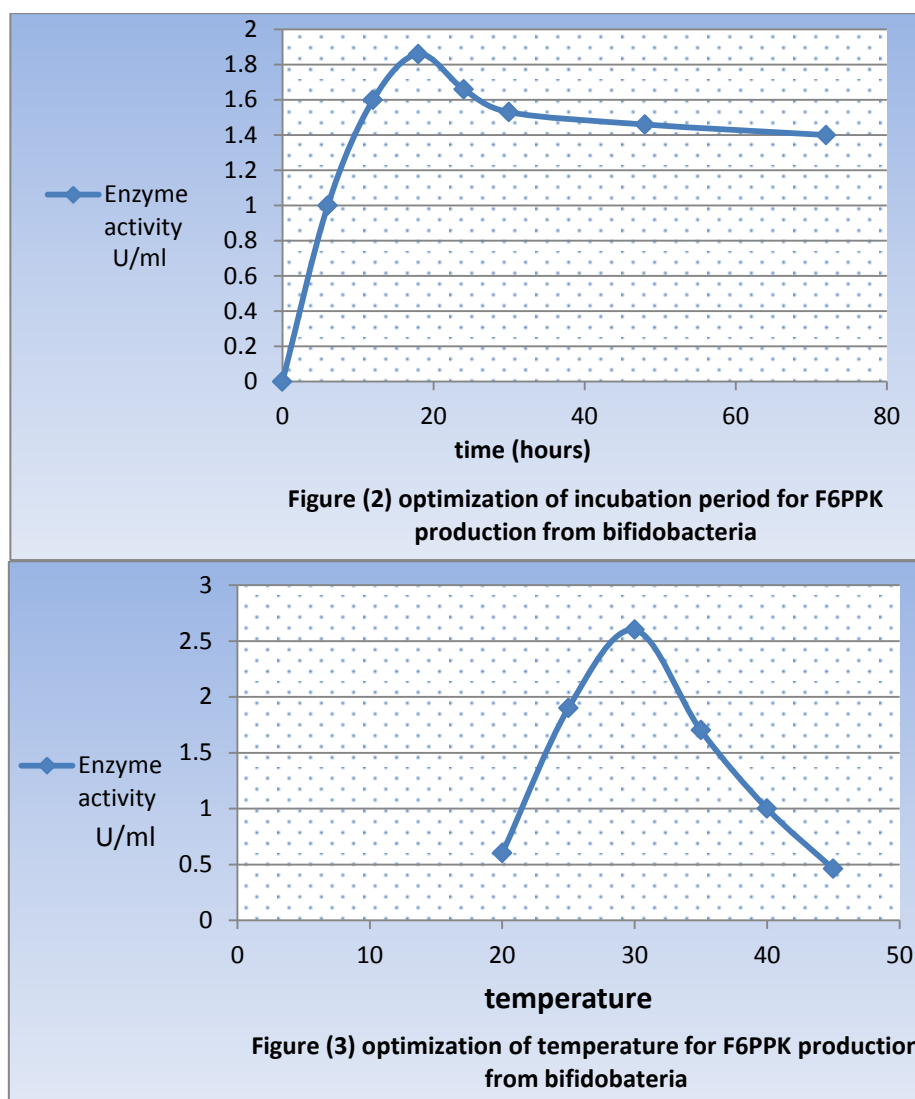
Results and Discussion

Twenty five samples out of the one hundred samples given a positive results as bifidobacteria . This samples had been grown on both MRS and LCL media and then submitted for identification . The selectivity of this media did not affect the growth of isolates, the colonies appear as round , white to cream in color , approximately 2 mm in diameter . grown of bifidobacteria on MRS agar and LCL agar was associated with grown of lactobacillus and candida species . So mupirocin susceptibility test was used to differentiated bifidobacteria from lactobacillus since first organism was resist to mupirocin while lactobacillus was sensitive. All of purified isolates were gram positive bacteria and given negative results for catalase, oxidase , IMVIC tests , and also generation of gas .

The F6PPK activity assay is commonly used as a definitive criterion for identification of bifidobacteria . F6PPK activity test was performed on all 25 samples which was considered to belonging to bifidobacteria. All of these samples given positive result for F6PPK activity test. The result was agreed with Zinedine and Faid 2007 who revealed that bifidobacteria can be distinguished on the basis of phosphoketolase production.

Optimization of conditions for production of F6PPK from bifidobacteria was determined as shown in the figures(1,2, and 3)





This result was agreed with optimal growth condition to growth of bifidobacteria . due to F6PPK considered as a key enzyme in the carbohydrate metabolic pathway of this bacteria, it is reach to maximum production during log phase and begin to decrease during stationary and decline phase. the results were also agreed with Hiederpoor *et al*, (2008) , who revealed that phosphoketolase assay is a common test for identification of bifidobacteria and all of strain that belonging to this genus given a positive result to F6PPK test.

Amplification of 16s rDNA and *xfp* Gene

Molecular detection of bifidobacteria by amplification of 16s rDNA and also detection of *xfp* gene was doing by PCR technique . molecular assay is an effective diagnostic tool for confirmation the isolates which considered as bifidobacteria by cultural and enzymatic method . A PCR product size was 914 bp length for 16s rDNA and 249 bp length for *xfp* gene. The result of amplification of 16s rDNA is show in figure (4).

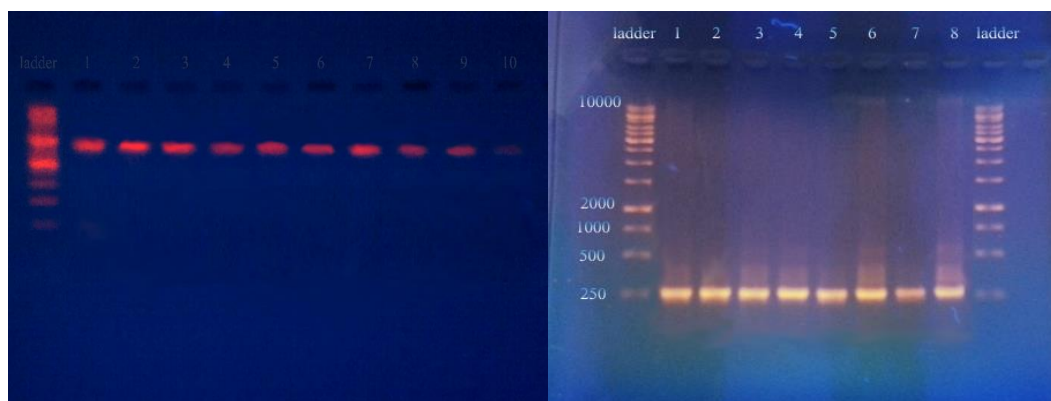


Figure (4) . On left Amplification of 16s rDNA by PCR technique (Amplicon size 914 bp) , line one DNA marker (100-3000 bp length) . On right Amplification of *xfp* gene by PCR technique, (Amplicon size 249 bp) line one and ten DNA marker (250-10000 bp length).

The result which shown in figure (4) was agreed with Denis and Stephane ,(2000) who revealed amplification of 16s rDNA with a specific primers pbi F1 and bpi r2 for all of the strain tested . 16S rDNA gene considered to be universally present in all bacteria and showed a high degree of sequence conservation the homology analysis in sequence of this gene given some interesting results for the phylogenetic analysis of the genus *Bifidobacterium* .

Conclusion

Isolation and characterization of bifidobacteria demonstrates that these bacteria are a good sources for probiotic strains . F6PPK assay considered to be a reliable test to identification of bifidobactertia from other probiotic bacteria such as lactobacilli , and used of CTAP method to disruption of cells membrane are a more rapid procedure than time consuming process by sonication . we determined the optimal conditions for F6PPK production that may be useful in growth of this bacteria at an industrial level.

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