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Theme

Optimizing the production of probiotic biomass in the presence of date extract

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LIST OF ABBREVIATIONS

AU	Arbitrary Unit
BLAST	Basic Local Alignment Search Tool
CFS	Cell Free Supernatants
CCD	Central Composite Design
DM	Dry Matter
FM	Fresh Matter
GAE	Gallic Acid Equivalent
LAB	Lactic Acid Bacteria
$LAPT_g$	Yeast Autolysed-Peptone-Tryptone-Tween 80-glucose
MRS	Man-Rogasa -Sharpe
OD	Optical Density
OFAT	One-Factor -At- a Time
OM	Organic Matter
PB	Plackett–Burman
TA	Titratable Acidity
UV-vis spectrophotometer	Ultra Violet-visible spectrophotometer

Introduction

During the last year, increasing interest has been observed on the role of natural products to promote human wellness and protection against the increased number of diseases. Among these natural substances, use of living microorganisms (probiotics) for human nutrition as effective nutraceuticals are widely used (Elmarzugi *et al.*, 2010). The industrial production of these bacteria is becoming more important. As probiotic microorganisms need a rich medium, it is necessary to search some alternative organic substrates for growth and production because of the existing economic recession and increasing cost of substrates (Manickavasagan *et al.*, 2012).

The agricultural and agro-industrial activities generate important amount of waste which constitute a sure nuisance for the environment (Kaidi & Touzi., 2001). However, these wastes are rich in sugars due to their organic nature, are easily assimilated by microorganisms and hence, make them potential substrates for exploitation as raw materials in the production of industrially relevant compounds through microbial conversion (Jawad *et al.*, 2013). One of the agro-wastes currently causing pollution problems is wastes date fruit. Since this fruit (*Phoenix dactylifera*) contains high amounts of sucrose as well as reducing sugars (total sugars of 44-88%), especially glucose and fructose, it may be a potentially convenient and inexpensive substitute for the glucose required by the lactobacilli (Shahravy *et al.*, 2012).

The optimisation of fermentation conditions, particularly physical, and chemical parameters are of primary importance in the development of any fermentation process owing to their impact on the economy and practicability of the process. Thus, it is of primary importance to develop an effective medium for higher biomass production. Nutritional requirements can be carried out by conventional or statistical techniques (Mouffok *et al.*, 2012).

This study was conducted to optimize culture conditions for economic production of a probiotic bacterium, *Lactobacillus plantarum* G1, in which palm date juice was applied as a low-cost main carbon source. The effect of seven factors on bacterial growth was investigated using the Plackett–Burman (PB) experimental design.

II.1. Probiotic

II.1.1. Probiotic definition

The word probiotics was initially used as an antonym of the word antibiotic. It is derived from Greek words « pro » and « biotos » and translated as for life (Azizpour *et al.*, 2009; Florou-Paneri *et al.*, 2013). According to Roy Fuller's definition (1989), probiotics are "live microbial feed supplement which beneficially affects the host (animal or human) by improving its intestinal microbial balance" (Ajmal & Ahmed, 2009; Pithva *et al.*, 2012; Thantsha *et al.*, 2012). *Havenaar* and *Huis in't Veld* broadened this scope (1992) and associated a probiotic with the description: "A viable mono- or mixed culture of microorganisms which, applied to animal or man, beneficially affects the host by improving the properties of the indigenous microflora" (Rusch, 2002).

It is clear that a number of definitions of the term probiotic have been used over the years but the one derived by the Food and Agriculture Organization of the United Nations/World Health Organization and endorsed by the International Scientific Association for Probiotics and Prebiotics (ISAPP) as they are known today: 'live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (Soccol *et al.*, 2010; Venugopalan *et al.*, 2010; Grajek *et al.*, 2005) and is currently the most accepted definition of probiotics by scientists worldwide (Reid, 2006; Thantsha *et al.*, 2012).

Although the definition probiotics includes the term 'live microorganisms', there is increasing evidence suggesting that nonreplicating bacteria, extracts or bacterial cell wall components could also exert health potentials (Gueniche *et al.*, 2010; Hwang *et al.*, 2010; Lew and Liong, 2013).

There are subcategories of the general term probiotic (Sanders, 2009) which are:
- Probiotic drugs: treat and prevent disease.
- Probiotic foods: food ingredients and dietary supplements.
- Direct-fed microbials: probiotics for animal use.
- Designer probiotics: genetically modified. Generally, foods containing probiotic bacteria fall in the category of functional foods

II.1.2. History of Probiotics

The concept of probiotics evolved around 1900. A father of immunology, Elie Metchnikoff, the noble prize winning Russian Immunologist demonstrated in 1908 that the reason behind the long live of Bulgarian peasants was due to the consumption of milk products fermented with the bacillus which had decreased the toxicity of colonic micro flora thus established positive influence of colon micro flora (Azizpour *et al.*, 2009; Ajmal & Ahmed, 2009).

At this time Henry Tissier, a French paediatrician, observed that children with diarrhoea had in their stools a low number of bacteria characterized by a peculiar, Y shaped morphology. These "bifid" bacteria were, on the contrary, abundant in healthy children. He suggested that these bacteria could be administered to patients with diarrhoea to help restore a healthy gut flora. The works of Metchnikoff and Tissier were the first to make scientific suggestions concerning the probiotic use of bacteria (Report of a joint FAO/WHO, 2001). A lot of research on probiotics had been carried out after that time and that increasing steadily since then (Oyetayo & Oyetayo, 2005).

II.1.3. Common probiotic microorganisms

Over the years, many species of microorganisms have been used (Gibson *et al.*, 2007). The strains most frequently used as probiotics include lactic acid bacteria and *bifidobacteria*, which have long history of safe use and are Generally Recognized As Safe (GRAS) (Sanders, 2009; Song *et al.*, 2012) are isolated from traditional fermented products and the gut, faeces and breast milk of human subjects (Fontana *et al.*, 2013). A notable number of microbial species and genera have been known to exhibit functional characteristics typically associated with probiotic properties (Table 1) (Lew & Liong, 2013).

In general most probiotics are gram-positive, usually catalase-negative, rods with rounded ends, and occur in pairs, short, or long chains. They are non-flagellated, non-motile and non-spore-forming, and are intolerant to salt. Optimum growth temperature for most probiotics is 37°C. With regard to fermentation probiotics are either obligate homofermentative (ex. *Lactobacillus. acidophilus*, *Lb. helvelicas*), obligate heterofermentative (ex. *Lb. brevis, Lb. reuteri*), or facultative heterofermentative (ex. *Lb. casei, Lb. plantarum*) (Song *et al.*, 2012). During the fermentation period, the probiotic bacterium is able to produce some antimicrobial metabolites like lactic acid, diacetyl, hydrogen peroxide and bacteriocin (Kanmani *et al.*, 2011).

Table 1: Microorganisms associated with probiotic propreties (Lew & Liong, 2013).

Lactobacillus	Bifidobacterium	Enterococcus	Streptococcus	Lactococcus
Lact. acidophilus	*Bif. adolescentis*	*Ent. faecalis*	*Strep. thermophilus*	*L. lactis subsp. cremoris*
Lact. brevis	*Bif. animalis*	*Ent. faecium*		*L. lactis subsp. lactis*
Lact. casei	*Bif. breve*			
Lact. curvatus	*Bif. infantis*			
Lact. fermentum	*Bif. longum*			
Lact. gasseri	*Bif. thermophilum*			
Lact. johnsonii				
Lact. reuteri				
Lact. rhamnosus				
Lact. salivarius				

Propionibacterium	Yeast	Others
P. freudenreichii	*Kluyveromyces lactis*	*Leuconostoc mesenteroides*
P. freudenreichii subsp. shermanii	*Saccharomyces boulardii*	*Pediococcus acidilactici*
P. jensenii	*Saccharomyces cerevisiae*	

II.1.4. Criteria of Probiotic selection

Microbial strain has to fulfil a number of specific properties or criteria for it to be regarded as a probiotic. These criteria are classified into safety, performance and technological aspects (Table 2) and are further dependent on specific purpose of the strain and on the location for the expression of the specific property (Thantsha *et al.*, 2012).

Table 2: Probiotic criteria for selection

Safety aspect	- probiotic microorganisms should not be pathogenic - no ability to transfer antibiotic resistance genes - be able to maintain genetic stability (Grajek *et al.*, 2005)
technological aspects	- Strains should be capable of being prepared on a large scale (Thantsha *et al.*, 2012) - They possess good sensorial properties, fermentative activity - good survival during freeze-drying or spray-drying - proper growth and viability in food products - phage resistance and high stability during long-term storage - Resistance to digestive enzymes (Grajek *et al.*, 2005; Azizpour *et al.*, 2009) - antimicrobial activity - tolerance to food additives and stability in the food matrix (Soccol *et al.*, 2010)
functional and physiological aspects	-Human specific organism -Adherence to the intestinal epithelium and colonization of the lumen of the tract and are expected to strongly interfere with the adhesion of pathogenic bacteria (Ohashi &Ushida, 2009; Azizpour *et al.*, 2009) -Production of antimicrobial substances (Lew and Liong, 2013) -immune modulatory activity (O'Hara & Shanahan, 2007) -It must have antimutagenic and anticarcinogenic properties and not promote inflammation inindividuals (Thantsha *et al.*, 2012) -withstand transit through the GI tract -stabilize the intestinal microflora and be associated with health benefits (Parvez *et al.*, 2006; Thantsha *et al.*, 2012)
Other aspect	- Capability for easy proliferation in vivo. - Capability for easy proliferation in vitro (Thantsha, 2012).

II.1.5. Mechanism of probiotic action

Probiotics have multiple and diverse effects on the host. The main mechanisms of action of probiotic bacteria by which they improve mucosal defenses of the gastrointestinal tract include:

II.1.5.1 Competitive Exclusion Along the Epithelium

Binding to intestinal epithelium is one of the determinants in establishing the efficacy of a probiotic (Michail, 2005). Several probiotic bacteria including *Bifidobacteria* and *Lactobacilli* adhere to mucosal tissue in a strain specific manner. This limits nutrient availability to other bacteria, enhances the intestinal persistence of the probiotic bacteria, and limits pathogen access to the epithelium surface structures (O'Hara & Shanahan, 2007).

The probiotics block the colonization of pathogenic bacteria by decreasing luminal pH, inhibiting bacterial invasion and adhesion to epithelial cells and producing antimicrobial compounds such as bacteriocins and defensins, organic acids and hydrogen peroxide (Florou Paneri *et al.*, 2013). For example, *Lb* GG and *Lb plantarum* 299V competitively inhibit the attachment of enterohemorrhagic *Escherichia coli* 0157H7 to HT-29 cells. The consumption of monosaccharides by a probiotic may reduce the growth of *Clostridium difficile*, which is dependent on monosaccharides for growth (Michail, 2005).

II.1.5.2. Enhancement of Epithelial Barrier Function

Probiotic bacteria can enhance barrier function by different mechanisms (Michail, 2005). These include the induction of mucin secretion, the maintenance or enhancement of cytoskeletal and tight junction protein phosphorylation, the restoration of chloride secretion, and the augmentation of transepithelial resistance (O'Hara & Shanahan, 2007; Florou-Paneri *et al.*,

2013). Probiotic bacteria such as *Streptococcus thermophilus* and *Lb acidophilus* enhance activation of tight junction proteins avoiding the development of a leaky intestine (Michail, 2005). The enhancement of mucosal barrier function may be an important mechanism by which probiotics benefit the host in various diseases such as Type 1 diabetes (Florou-Paneri *et al.*, 2013).

II.1.5.3. Immunomodulation

There may be intrinsic differences in how epithelial cells sense commensal or probiotic bacteria versus pathogenic bacteria at the level of signal transduction pathways and cytokine production (Ng *et al.*, 2008). Probiotic bacteria may affect the epithelial cells, the dendritic cells, the monocytes / macrophages and the various types of lymphocytes (Natural killer cells, T-cells and T-cell redistribution) directly or secondarily. Moreover, the effects of probiotics on B-lymphocytes and antibody production resulted in an increase in IgA secretion and the enhancement of response to vaccination (Florou-Paneri *et al.*, 2013).

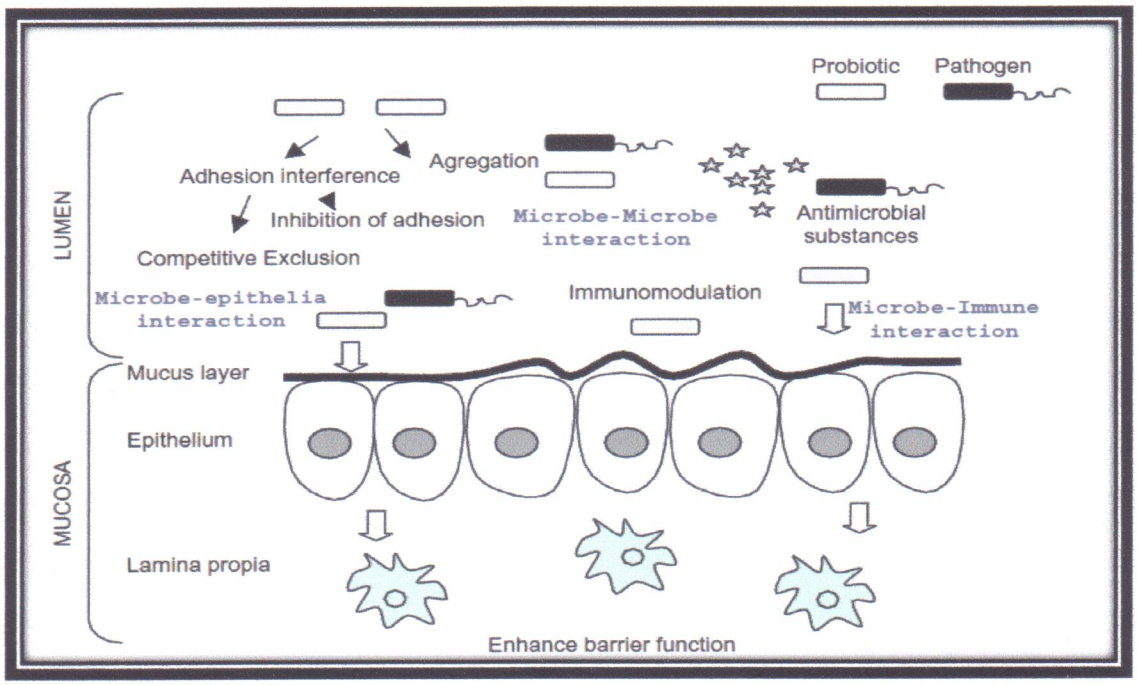

Figure 1: Mechanisms of probiotics against pathogen infection. 1. antimicrobial substances against pathogens. 2. Immunomodulation. 3. Improvement of barrier function. 4. adhesion: competitive inhibition with pathogenic bacteria, inhibition and displacement of pathogen's adhesion. 5. aggregation and coaggregation with pathogens (Watson & Preedy, 2010).

II.1.6. Beneficial effects of probiotics

Probiotics provide beneficial effects on the host's health by affecting the intestinal microflora. The benefits attributed to probiotics can either be nutritional or therapeutic (Ohashi & Ushida, 2009; Thantsha *et al.*, 2012).

II.1.6.1. Nutritional benefits

Probiotics are commonly consumed as part of fermented foods such as fermented dairy products like yogurt, kefir, kumis cheese and fermented non-dairy products like soy, yogurt, fruit, vegetable and malt beverages (Thantsha *et al.*, 2012). They used in food industry, imparting flavor, texture and possessing preservative properties (Grajek *et al.*, 2005). Ingestion of probiotics is associated with improved production of riboflavin, niacin, thiamine, vitamin B6, vitamin B12 and folic acid. Probiotics play a role in increasing bioavailability of calcium, iron, manganese, copper, phosphorous and increase the digestibility of protein and fat in yogurt (Thantsha *et al.*, 2012).

II.1.6.2. Therapeutic benefits

- **Gastric and intestinal tract effect of probiotics**

There are a number of studies in humans that suggest that lactic acid bacteria can decrease the incidence, duration and severity of some gastric and intestinal illnesses. Probiotics are also used to treat other types of diarrhea, such as traveler's diarrhea caused by enterotoxigenic *Escherichia coli*, acute rotavirus diarrhea and antibiotic-associated diarrhea (Ohashi & Ushida, 2009). Studies show that different probiotic microorganisms, *Lb. rhamnosus* GG, *Lb. reuteri*, *B. Lactis*, *S. thermophilus* 065 and *Saccharomyces boulardii*, exert protective effects or shorten the duration of acute diarrhea when induced by rotavirus (Watson & Preedy, 2010; Corcionivoschi *et al.*, 2010).

- **Colon Cancer**

Experimental studies clearly demonstrate a protective effect of probiotics (*Bifidobacterium bifidum* and *Lb. acidophilus* against colon cancer. They can prevent the establishment, and metastasis of transplantable and chemically induced tumors (Charalampopoulos & Rastall., 2009) by decreasing the levels of enzymes such as β-glucuronidase, and nitroreductase responsible for activation of procarcinogens into carcinogens and consequently decrease the risk of tumour development (Pithva *et al.*, 2012).

- **Probiotics in the Management of Allergy**

Weston *et al.* and Kalliomaki *et al.* demonstrated that probiotics may be effective in reducing the severity of atopic dermatitis and prevent atopic disease in children at high risk, respectively (Upadhyay & Moudgal, 2012). Probiotics have been documented to exert dermal potentials such as improving atopic eczema ,healing of burn and scars and skin-rejuvenating properties (Lew & Liong, 2013).

- **Prevention of Infections in the Urogenital Fields**

Bacterial vaginosis associated with disruption of vaginal microflora has been suggested to allow invasion of various pathogenic microorganisms of sexually transmitted diseases (STD). Attempts to normalize vaginal bacterial flora by probiotics to inhibit the spread of STDs such as AIDS are a future task (Watson & Preedy, 2010). The potential role of genetically engineered probiotics as vaginal microbicides to prevent HIV is provocative; however, it is in the early stages of development and further research in this field is needed (Upadhyay & Moudgal, 2012).

- **Dental Caries**

The use of probiotics and molecular genetics to replace and displace cariogenic bacteria with noncariogenic bacteria to treat the caries causing pathogens has shown promising results. Early studies utilized recombinant strain of *S. mutans* expressing urease, which was shown to reduce the cariogenicity of plaque in an animal model (Agrawal *et al.*, 2012).

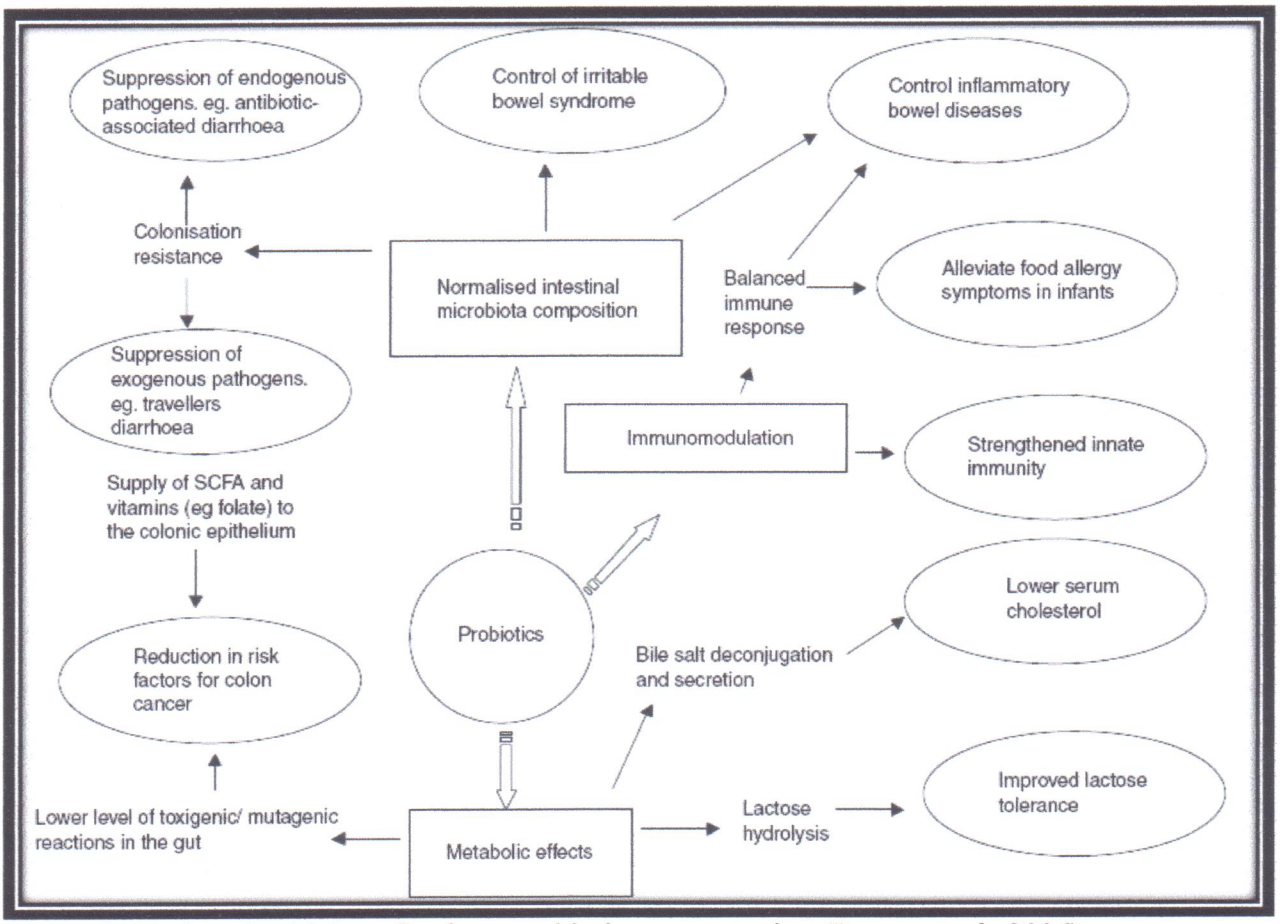

Figure 2: Various health benefits from probiotics consumption (Parvez *et al.*, 2006).

II.2.Technology of probiotic production and optimization

Industrial production of microorganisms as biomass for varied applications is practiced over the years particularly with reference to yeasts that serve as biocatalysts or source of products in bakeries, breweries; lactic acid bacteria as starter cultures for use in dairy product manufacture; probiotic for application in dairy industries (Chandrasekaran & Bahkali, 2013).

The Increasing demand for products containing probiotic microorganisms from health-conscious consumers need to establish effective fermentation techniques for probiotic biomass production. Medium formulation, fermentation parameters and fermentor design play important roles to ensure optimal results (Chang & Liew, 2012).Until now, technological developments for the production of probiotics have focused on approaches to obtain as many cells as technically and/or economically possible. Clearly, in addition to high cell numbers, cell physiology is crucial to ensure that cells are well-suited to survival during downstream processes and that they exhibit high functionality (Lacroix &Yildirim, 2007).

Production of probiotics should be based on the microbial criteria, (Kosin & Rakshit, 2006) the cost of nutrient substrates and the processes used in their production. In addition, parameters for scale optimisation and amplification are necessary. According to Schmidell, different phases of the process must be evaluated, such as the kinetics of growth, and primary or secondary metabolite production, as well as separation, recovery and formulation of the products. The fermentative process requires monitoring parameters of the culture system as a function of the fermentation time (Pancheniak *et al.*, 2012).

Probiotic production has almost exclusively been carried out using conventional batch fermentation and suspended cultures, in some cases combined with the use of sublethal stresses to enhance cell viability, the addition of protectants or microencapsulation to provide cell

protection. However, other less conventional fermentation technologies, such as continuous culture and immobilized cell systems, could have potential for enhancing the performance of these fastidious organisms. These technologies might be employed to develop strains with improved physiology and functionality in the gut and to enlarge the range of commercially available probiotics, as well as expanding product applications (Lacroix &Yildirim, 2007).

II.2.1. Bioprocess optimization

Optimization theory consists of a body of numerical methods for finding and identifying the best candidate from a collection of alternatives without having to explicitly evaluate all possible alternatives (Chen *et al.*, 2004).

The biomass and secondary metabolites production was affected by many typical fermentation factors such as composition of the medium: carbon, nitrogen ratio, pH of the culture medium, temperature, type of aeration, inducers and presence of some precursors. However to estimate practically the interactions of the conditions and to establish better conditions by interrelating all the factors considered numerous experiments had to be carried out with all possible combinations of parameters, which was not practical and possible (Bhima *et al.*, 2012). As a result during bioreactor cultivation of lactic acid bacteria in industrial scale, there are two main problems during process namely: low cell growth rate and high lactate production. These both together resulted in low cell mass production (Elmarzugi *et al.*, 2010). Consequently the optimization of the culture medium is one of the most important steps in the development of an economical production process that produces a quality probiotic product (Gao *et al.,* 2009).

Recent developments in the area of optimization of fermentation conditions have resulted in a significant increase in production yields, making them more commercially attractive (Rodrigues *et al.*, 2006). Maximizing productivity or minimizing production costs demands the use of process-optimization strategies that involve multiple factors (Mukherjee *et al.*, 2006). Since the cost of culture medium has a remarkable impact on the mass production of probiotics, the optimization of growth conditions, substitution with low-price nutrient ingredients and simplification of medium are vital for their economical production (Shahravy *et al.*, 2012).

II.2.1.1. Media design

As probiotic organisms are fastidious with respect to nutrient requirements, a rich medium is required for good growth. Proper design of the media does affect the performance of microorganisms in optimizing the biomass production (Sreekumar & Krishnan, 2010; Djukic-Vukovic *et al.*, 2012).

The main objective of media optimization is to produce maximum yield of product or biomass per gram of substrate used (Sreekumar & Krishnan, 2010). The standard growth media employed to cultivate lactic acid bacteria (LAB) such as De Man-Rogosa-Sharpe (MRS) and yeast autolysed-peptone-tryptone-Tween 80-glucose ($LAPT_g$) are expensive for industrial application. Therefore, it is of primary interest to reduce the cost of the growth media employed for industrial production of biomass and metabolites, which can be achieved by decreasing the concentrations of nutrients at an indispensable minimum or by using low-cost components (Tomas *et al.*, 2010) such as starch or glycogen agriculture or food processing wastes. However, most probiotic or bacteriocinogenic strains are not amylolytic and the use of carbohydrates, as glycogen from mussel processing wastes, requires a previous step of enzymatic hydrolysis. Therefore, the probiotics culture could be proliferated in a mixed-culture system where a bacteriocin-resistant amylolitic strain could facilitate the degradation of the complex polysaccharides (Pintado *et al.,* 2003).

LAB have specific growth factors requirement. In practice, requirements for growth factors are satisfied by the addition of nitrogen sources. Among a variety of nitrogen sources, yeast extract (YE) is the best one due to its high content of nitrogen compounds, abundant purine, pyrimidine bases and vitamins (Liu et *al.*, 2010). A number of substances are well known to improve the growth of probiotic bacteria,eg the supplementation of milk with a combination of casitone, casein hydrolysate (Chen *et al.*, 2004).

Several studies focused on the optimization of the growth medium of probiotic strain, Abd Malek *et al.*, (2010) studied probiotic production from synthetic medium by *Lb. salivarius* WICC-BO8, they found that the optimal medium which gives highest biomass was composed of (g L^{-1}): glucose, 20; yeast extract, 20.0 and peptone, 35. The maximum biomass of 7.57 g/l was obtained with controlled pH bioreactor. Pathak *et al.,* (2012) conducted a study to modify de Man Rogosa Sharpe culture medium (MRS), using plant seed powders for the growth of probiotic starter culture of *Lb. lactis,* they found that the growth of probiotic increased significantly when compared with standard MRS medium.

Combinative interactions of parameters with the growth and required metabolite production by microorganisms are legion. Therefore optimum processes should be designed using effectual experimental methods (Ramakrishnan *et al.*, 2012). Many techniques in optimising culture medium and cultivation conditions are available (Chen *et al.*, 2010) According to the number of the factors to be investigated at a time, the experimental design can be classified into two categories: one-factor-at-a-time design (single-factor design) and factorial design (multiple-factor design) (Wang & Wan, 2009).

II.2.1.1.1. Conventional methods

Most industrial media are composed by complex components such as yeast extract, peptone, soy bean meal, casein, etc. Therefore, it is difficult to determine the C/N ratio of this components. One factor at a time for determination of C/N and media composition, a typical method used for optimizing a multivariable system (Cho *et al.,* 2008). In the conventional scaling-up approach one-factor at a time (OFAT), various nutritional and physical parameters were optimized by maintaining all factors at a constant level in the basal medium, except the one under study. Each subsequent factor was examined after taking into account the previously optimized factor. This approach is simplest to implement, and primarily helps in selection of significant parameters (Singh *et al.,* 2011). Some researchers have articulated a role for OFAT and demonstrated that it has some advantages under some conditions.

Daniel, (1973) suggested that OFAT may be preferred when an experimenter wishes to react more quickly to data and can be safely used in those cases in which factor effects are three or four times the standard deviation due to pure experimental error. Friedman and Savage, (1974) suggested that an OFAT approach might be preferred over balanced factorial plans when the experimenter seeks an optimum within a system likely to contain interactions. They suggested that OFAT might offer advantages because it concentrates observations in regions likely to contain the optimum.

For example, Kumar *et al.*, (2012) investigated the effects of physical Parameters: temperature (15-45°C), pH (4.0-7.0), incubation time (up to 48h) and inoculum size (0.7-2.0 O.D) on bacteriocin production by *Lactobacillus casei* LA-1 using one-factor-at-a-time design, they found that all the factors have a significant effect on bacteriocin production. Bacteriocin production of 2,844 AU/ml was obtained at temperature 37°C, pH 6.7 and inoculum size 1.8 O.D at an incubation time of 20h.

This method is laborious, may lead to unreliable and wrong conclusions and also extremely time consuming and expensive (Patil *et al.*, 2011; Anisha *et al.*, 2008), often fail to

identify the optimal conditions for the bioprocess and can lead to misinterpretation of results because interactions between different factors are neglected. Moreover, they require a considerable amount of work (Gao *et al.*, 2009; Kiruthika *et al.*, 2001; Gupta *et al.*, 2013).

On the other hand carrying out experiments with every possible factorial combination of the test variables is impractical because of the large number of experiments required. In the first screening, it is recommended to evaluate the result and estimate the main effects according to a linear model. After this evaluation, the variables that have the largest influence on the result are selected for new studies. Thus, a large number of experimental variables can be investigated without having to increase the number of experiments to the extreme (Rodrigues *et al.*, 2006; Rajendran *et al.*, 2007; Kanmani *et al.,* 2011).

To tackle these problems and make the optimization process easier, a statistical optimization strategy has been used by various investigators (Mukherjee *et al.,* 2006).

II.2.1.1.2. Statistics methods

Statistically designed experiments have been employed for over half a century across numerous industries and were first described in The Design of Experiments by the British statistician and biologist Sir Ronald Fisher (Altekar *et al.*, 2006). Statistical methods provide an alternative methodology to optimize a particular process by considering the mutual interactions among the variables and give an estimate of combined effect of these variables on final results (Kiruthika *et al.,* 2011; Shahravy *et al.,* 2012).

Statistical methods offer several advantages over conventional methods in being rapid and reliable and that shortlists significant nutrient, helps understanding the interactions among the nutrients at various concentrations and reduces the total number of experiments tremendously resulting in saving time and material (Kaur *et al.*, 2013), minimize the error in determining the effect of parameters and also enhances finding out the optimal conditions by establishing the relationship between factors and predicted responses (Sreekumar and Krishnan, 2012).
Experimental factorial designs and response surface methodology are statistical methodologies used to efficiently optimize several bioprocesses, being also frequently employed to determine multiple responses. In these situations, the optimal values obtained for one response may not be optimal for other responses, because the optimization of a dependent variable is obtained at the expense of the other. The desirability function is a useful tool to determine the levels of the controlled factors for which the responses simultaneously reach the minimal or maximal expected values (Tomas *et al.,* 2010).

Optimization process involves three major steps, which are performing the statistically designed experiments, estimating the coefficients in a mathematical model and predicting the response and checking the adequacy of the model (Annadurai *et al.*, 2008).

II.2.1.1.2.1. Screening design

- **Plackett-Burman design**

Is a two-level factorial design, which identifies the critical physicochemical parameters by screening *N* variables in *N*+ 1 experiment (Anisha *et al.*, 2008). Each factor was prepared in two levels: (-1) for low level and (+1) for high level. It is very useful in identifying the important nutrients for cell growth (Cho *et al.,* 2008).

Plackett- Burman design can be used to find the significant variables in a system and allow them to be ranked in order of importance and to decide which one is to be investigated further so as to determine the optimum values (Sreekumar and Krishnan, 2010), but it does not consider the interaction effect among the variables (Anisha *et al.*, 2008; Elsanhoty *et al.,* 2012)

and cannot detect the optimum level of factors, also could offer indications and trends regarding the effects of each factor in a small number of experiments.

The bi-factorial Plackett-Burman model permits the screening of the significant factors from a number of variables in the process, and this model is useful in the preliminary studies in which the main objective is to select variables which could be fixed or eliminated during the further process optimization (Paraschive *et al.*, 2012). The results of the analysis of this design are presented in the form of a Pareto chart (Polak-Berecka *et al.,* 2011)The variables that are found significant in the initial screening can be further optimized using response surface methodology (RSM) (Anisha *et al.*, 2008).

Elsanhoty *et al.*, (2012) used Plackett–Burman design to Screen medium components for carotenoid production using date (*Phoenix dactylifera*) wastes as source of Sugar. The results showed that peptone, K_2HPO_4, sodium acetate and date juice as main components affecting carotenoids production.

- **Taguchi design**

Taguchi design, which is a fractional factorial design using orthogonal array, allows the effects of many factors with two or more levels on a response, to be studied in a relatively small number of runs. In addition, the orthogonal array facilitates the analysis of the design. When used properly, Taguchi design may provide a powerful and efficient method to find an optimal combination of factor levels that may achieve optimum. Usually, with the aid of range analysis, analysis of variance or analysis of signal-to-noise ratio, the key factors that have significant effects on a response can be identified and the best factor levels for a given process can be determined from the pre-determined factor levels (Wang &Wan, 2009).

Bhima *et al.*, (2012) studied the effects of eight factors on biomass production of probiotic yeast S*accharomyces cererivisiae* using Taguchi design. Based on the analysis of the experimental results, they determined that *Prosopis* pods, pH, inoculum size, urea and boric acid were significant factors for yeast biomass production. Phosphoric acid, temperature and yeast extract showed negligible influences on the yeast biomass production. The optimal biomass was 7.848 g/l on dry weight basis. However, the true optimal factor levels may not be guaranteed using Taguchi design, because the true optimal factor levels may be different from the corresponding pre-determined factor levels (Wang &Wan, 2009).

II.2.1.1.2.2. Response surface methodology

To analyze the effect of various factors in better way, a number of statistical approaches with response surface methodology are attempted for the optimization of bioprocess (Singh *et al.*, 2011).

Response surface methodology (RSM) is a highly developed and widely practiced set of techniques. The key ideas behind RSM were first published by Box and Wilson (1951) is a much more efficient technique for optimization (Frey and Wang, 2006). RSM is a combination of statistical and mathematical techniques useful for optimization of bioprocesses, and it can be used to evaluate the effect of several factors that influence the responses by varying them simultaneously in limited number of experiments (Preetha *et al.*, 2007). This methodology has been utilized successfully to optimize culture conditions and medium composition (Sifour *et al.*, 2010, Brinques *et al.*, 2010; Shahravy *et al.*, 2012), conditions of enzymatic hydrolysis, and parameters of food preservation (He *et al.*, 2004), improve fermentation processes (Rahim *et al.*, 2007) and for developing new products (Preetha *et al.*, 2007). It uses quantitative data to simultaneously determine and solve multivariate equations, graphically represented as response surfaces. It usually contains three stages:

(1) Design of experiments
(2) Response surface modelling through regression
(3) Optimization (Chen *et al.*, 2004).

Response surface methodology can be implemented to estimate a polynomial model representing the effect of significant factors on viable cell counts in the probiotic products, as well as to optimize the process variables (Shahravy *et al.*, 2012). Response surface methodology has eliminated the drawbacks of classical methods and has proved to be powerful and useful for the optimization of the target metabolites production (Gao *et al.*, 2009).

- **Box–Behnken design**

Is a three-level fractional factorial design developed by Box and Behnken (Wang &Wan, 2009), a spherical, revolving RSM design that consists of a central point and the middle points of the edges of the cube circumscribed on the sphere. The design leads to the generation of contour plots by linear or quadratic effects of key variables and a model equation is derived that fits the experimental data to calculate the optimal response of the system (Gupta *et al.*, 2013; Singh *et al.*, 2011). Box–Behnken design provides an economical alternative to the central composite design, because it has less factor levels than the central composite design and does not contain extreme high or extreme low levels (Wang &Wan, 2009).

Pereira *et al.*, (2010) used Plackett–Burmam design and Box-Behnken design to develop a medium based on corn steep liquor (CSL) and other low-cost nutrient sources for high-performance very high gravity (VHG) ethanol fermentations by *Saccharomyces cerevisiae*. They found that the optimized medium composed of (g/ l: CSL 44.3, urea 2.3, $MgSO_4$-$7H_2O$ 3.8 and $CuSO_4$-$5H_2O$ 0.03), they suggested that their study represent valuable tools for the development of cost-effective industrial fermentation media.

- **Central composite design (CCD)**

One of the most commonly used designs for response surface optimisation (Stephenie *et al.*, 2007), where experiments are added to the factorial design after nonlinear behaviour is detected (Altekar *et al.*, 2006), CCD has three groups of design points: two-level-factorial or fractional-factorial design points, axial points (sometimes called 'star' points) and centre points. CCDs are designed to estimate the coefficients of a quadratic model (Preetha *et al.*, 2006).

Bevilacqua *et al.*, (2008) developed a four variables- five Central Composite Design to study the effects of pH, yeast extract, carbohydrates, di-ammonium hydrogen citrate and pH on biomass production and acidifying ability of a probiotic *Lb. plantarum* strain, isolated from table olives, in a batch system. The results showed that the biomass production was maximal at pH 6.0 and lactose 20g l^{-1}.

- **A face-centred central composite design**

Is a modified CCD provided a good alternative for CCD, where the added experiments lie on the faces of the space formed by the factorial design (Preetha *et al.*, 2006). Tari *et al.*, (2009) studied the optimization of the associative growth of novel yoghurt cultures (*Streptococcus thermophilus* 95/2 and *Lb. delbrueckii* ssp. *bulgaricus* 77) in the production of biomass, β-galactosidase and lactic acid using face-centred central composite design. The key medium components for their optimization were to use these cultures in a ratio of 1.66:1.62 (St 95/2:Lb 77) ,(5%) whey, (4%) corn steep liquor, (2%) potassium phosphate and (2%) peptone at 43 °C for 8 h. The associative growth provided 6.4% and 39% more β-galactosidase activity and 8.73% and 44% more lactic acid.

Optimization through factorial design and response surface analysis is a common practice in biotechnology and various research workers have applied these techniques for the optimization of culture conditions (table 3).

Table 3: Application of optimization methods to improve biomass and metabolites production.

microorganisms	Experimental design	Optimisation purpose	yield	reference
Bifidobacterium pseudocatenulatum G4	2^3 full factorial design+ face-centered central composite design	Improving the growth in Milk Medium	biomass increase by 3.329 log units	Stephenie *et al.* (2007)
Lactococcus lactis subsp. *lactis* LLC518	Plackett-Burman design, steepest ascent design and central composite design	production of lacticin LLC518	86.3% increase in the production	Meng *et al.* (2012)
Enterococcus faecium MC13	2 level factorial design, steepest ascent path and central composite design	Bacteriocin production	Productivity and total viable cell were two times higher than MRS medium	Kanmani *et al.* (2010)
Lactococcus lactis 53 and *Streptococcus thermophilus A*	steepest ascent and Plackett-Burman design	Production of Biosurfactant	Productivity was 1.6 times higher than MRS medium	Rodrigues *et al.* (2006)
Pseudomonas MCCB 103	one-variable-at-a-time and central composite design	production of an aquaculture probiotic	19% increase in biomass and fivefold increase in the antagonistic compound production	Preetha *et al.* (2007)
Lactobacillus plantarum Pi06	Taguchi array design and the Box-Behnken method	biomass production	biomass yield increase by approximately 107%	Hwang *et al.* (2012)
Starter culture	one factor at a time and 2^4 full-factorial central composite design	maximizing kefir grain biomass yield	biomass increased by 81.34%	Ghasemlou *et al.* (2012)
L. plantarum MTCC 1407	Plackett–Burman design and response surface methodology	enhancement of tannase production	2.0 fold increase in tannase production	Natarajan *et al.* (2012)
Lb. fermentum	Plackett–Burman design and central composite design	β-galactosidase production	productivity was improved by 14.60 folds.	Sriphannam *et al.* (2012)

Even if optimum production is obtained using optimal media and culture conditions, the production process is still incomplete without an efficient and economical means for the recovery of the products. Thus, one important factor determining the feasibility of a production process on a commercial scale is the availability of suitable and economic recovery and downstream procedures. For many biotechnological products, the downstream processing costs account for 60% of the total production costs (Gupta *et al.*, 2013).

II.3. valorization of industrial wastes and by-products

The food and agricultural industries produce large volumes of wastes annually worldwide (Jawad *et al.*, 2013), causing a serious disposal problem to in the environment, and also interfere with all natural processes of ecosystems (Chandrasekaran, 2012).This is especially problematic in countries where the economy is largely based on agriculture and where the farming practice is very intensive. Currently, these agro-wastes are either allowed to decay naturally on the fields or are burnt. However, these wastes are rich in sugars due to their organic nature, are easily assimilated by microorganisms and hence, make them potential substrates for exploitation as raw materials in the production of industrially relevant compounds through microbial conversion. In

addition, the reutilization of biological wastes is of great interest since, due to legislation and environmental reasons; the industry is increasingly being forced to find an alternative use for its residual matter (Jawad *et al.*, 2013).

II.3.1. Concept of valorization

Valorization is a relatively new concept in the field of industrial residues management and promotes the principle of sustainable development. Valorization is the process of value creation from knowledge by making a product or waste or less-value matter (or energy) suitable and or available for economic and / or social use. One of the valorization objectives regarding food processing by-products, wastes, and effluents is recovery of fine chemicals and the production of precious metabolites via chemical and biotechnological processes translating them into competitive products, services, processes, or new commercial activities (Chandrasekaran, 2012).

The food wastes can be classified into different categories, such as crop waste and residues; fruits and vegetables by-products; sugar, starch and confectionary industry by-products; oil industry by-products; grain and legumes by-products; distilleries and breweries by-products (Ajila *et al.*, 2012; John *et al.*,2007).This waste and effluents typically consist of high amounts of proteins, sugars and lipids along with particular aromatic and aliphatic compounds and, therefore, they could be cheap and abundant sources of fine chemicals and biomaterials (Federici *et al.*, 2009). There are different ways of utilization of by-products from food processing industries. This ways are mentioned in (figure 3).

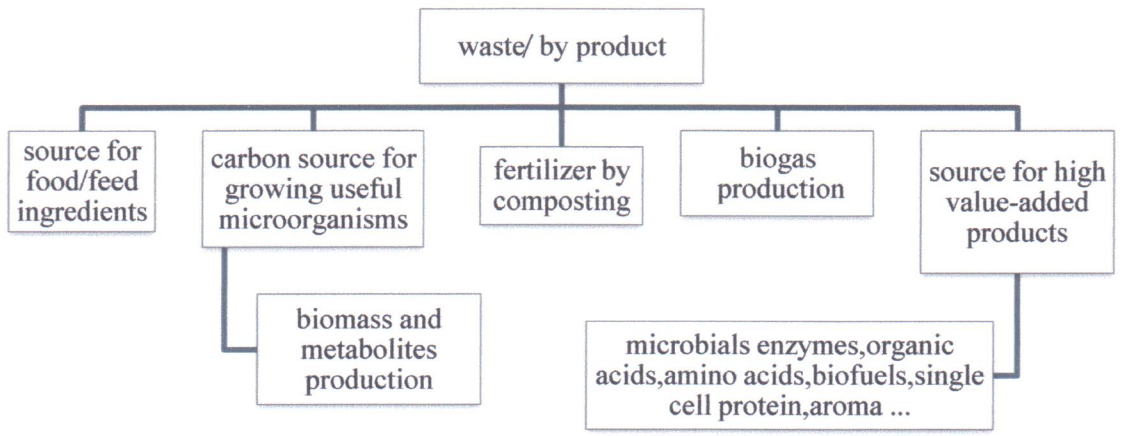

Figure 3: Different ways of valorization of wastes / by-products.

The production economy of every microbial metabolite is governed by three basic factors: initial raw material costs; availability of suitable and economic production and recovery procedures; and the product yield of the producer microorganisms (Saharan *et al.*, 2011; Baliga *et al.*, 2011). To reduce this cost, it is desirable to use low-cost raw materials. One possibility explored extensively is the use of cheap and agro-based raw materials as substrates for production. Several researchers reported the use of cheap raw materials as a source for high added products that are represented in (table 4).

Table 4: waste / by-product as carbon source for growing useful microorganisms.

wastes and by-products	microorganisms	High value added products	reference
cornstarch and soy flour	-*Lactococcus lactis* -*Streptococcus thermophilus* -*B. licheniformis*	Biosurfactants protease RG1	Saharan *et al.* (2012)
-Tea waste -Sugarcane molasses -Mango peel waste	-*Lactobacillus plantarum* -*Lactobacillus delbrueckii* -indigenous microorganisms	lactic acid production	-Gowdhaman *et al.* (2012) -Farooq *et al.* (2012) -Jawada *et al.* (2012)
-starchy waste: potato pulp, potato fruitwater, pea pulp, pea-fruitwater as wastes,The draff residue, Bread residue -corn steep liquor	*Bacillus caldolyticus* DSM 405	α-amylase and proteases	Jamrath *et al.* (2011)
-bakery waste : pastry waste, cakepieces -waste bread	-*Aspergillus awamori* (ATCC 14331) and *Actinobacillus succinogenes* (ATCC55618) -*Actinobacillus succinogenes*	Succinic acid	-Zhang *et al.* (2012) - Leung *et al.* (2012)
-Waste water from lettuce and apple processing. -Waste glycerol from biofuel production	-*Rhodotorula glutinis* -*Rhodotorula sporobolomyces*	Biomass lipid and carotenoid production	-Schneider *et al.* (2012) -Petrik *et al.* (2013)
Sugarcane bagasse, groundnut hulls rice husks.	*Aspergillus niger* and *Aspergillus niger* MTCC	Production of citric acid	John *et al.* (2012)
Cassava bagasse, apple pomace, amaranth and soya bean.	*C. fimbriata*	Fruity aroma production	Mussatto *et al.* (2012)
Orange peel, lemon peel, apple peel, corncob, rice straw, and wheat bran.	*Aspergillus niveus*	Pectin Lyase production	Maller *et al.* (2012)
Banana peels	-*Aspergillus niger* -*Bacillus subtilis* -*Sacchromyces cerevisiae R8*	Animal feed protein enrichment, amylase, Bio-ethanol, bio-methane	Jamal *et al.* (2012)
vegetal oils	*Candida lipolytica*	Singl Cell protein production	Begea *et al.* (2011)
Vegetable wastes : tomato, lemon, carrot and fennel	-	Extremophile biomass	Donato *et al.* (2011)
cheese whey	*L. lactis* 53 *S.thermophilus* A	Biosurfactants production	Rodrigues & Teixeira, (2008)
kitchen waste	*Lactobacillus sp,bacillus licheniformis,*yeast	Probiotic production	Yin *et al.* (2013)

II.3.2. Valorization of dates palm by-products and wastes by biotechnological processes

Date is a berry fruit of the palm tree (*Phoenix dactylifera*), mainly grown in Arabian countries and desert regions of other tropical countries (Tavakkoli *et al.*, 2012; Mukherjee *et al.*, 2006), has played an important role in the day-to-day life of the people for the last 7000 years (Chandrasekaran & Bahkali., 2013). The world production of dates has increased considerably during the last three decades and estimated more than 6.7 million tones (Manickavasagan *et al.*, 2012). Algeria produces different varieties of dates and the annual production is over 720,000 tons (Mouffok *et al.*, 2012), constituted of common dates and date wastes (Acourene & Ammouche, 2010).

Industrialization of dates has focused mainly on conventional processes, such as pitting, packaging, date pastes and animal feed. Biotechnological industrial processes using dates as raw materials are highly flexible and can accept most date cultivators. However the most important factors to be considered in selecting date cultivators suitable for the production process are the sugar content, price per ton and storage life of the dates (Jain *et al.*, 2011). Date wastes, in fact,

consist of cellulose, hemicelluloses, lignin and other compounds which could be directly utilized by fermentation microorganisms and used in many biological processes; however they were burned in farms causing a serious threat to the environment, besides contributing to a great loss of raw materials (Jain *et al.*, 2011; Chandrasekaran & Bahkali, 2013).

II.3.2.1. Nutritional value and biochemical composition

Dates are rich in certain nutrients and provide a good source of rapid energy, due to their high carbohydrate (glucose and fructose) content (70–80%). Moreover, date fruits contain fat (0.20–0.50%), protein (2.30– 5.60%), dietary fibre (6.40–11.50%), minerals (0.10–916 mg/100 g dry weight), potassium (2.5 times more than bananas), calcium, magnesium and iron, some vitamins (C, B1, B2, B3 and A) with very little or no starch (El Arem *et al.*, 2011; (Chandrasekaran & Bahkali, 2013). Besides nutritional value, date fruits are rich in phytochemicals such as phenolic compounds (Shahidi & Alasalvar, 2013; Benmeddour *et al.*,2013) Many studies have shown that date fruit has antioxidant, anti-mutagenic, anti-inflammatory, gastro-protective, hepato-protective, nephro-protective, anticancer, immune-stimulant activities etc (Tang *et al.*, 2013; Martín-Sánchez *et al.*,2013; Jain *et al.*, 2011).

II.3.2.2. Date palm fruits processing by-products/wastes and their valorization process

Several date processing industries are developing new processes to produce many products like date juice, date syrup, date dip, date honey, date jam, date paste and date vinegar from various date varieties (Jain *et al.*, 2011). Unfortunately, this progress of production is accompanied by a substantial increase of date losses during picking, storage or conditioning of the dates Elsanhoty *et al.*, 2012; Sanchez-Zapata *et al.*, 2011) Due to their inadequate texture (too soft), the lost dates, commonly named ''date by-products'', are not edible and are often discarded (Chandrasekaran & Bahkali, 2013; Elsanhoty *et al.*, (2012). Today and with the biotechnologies processes, it is possible to upgrade by-product and waste dates as well, and to put on the local and international market, a new generation of products with high values added (Boulal *et al.*, 2010).

According to one estimate, each date tree produces about 20 kg of dry leaves yearly. Other wastes such as date pits represent an average of 10% of the date fruits and are defined as the form that fell from the tree before maturity (Chandrasekaran & Bahkali, 2013); Cull dates represent an enormous global production rate about 30% of the date production. Consequently the definition of a cull date is rather flexible but is generally connected with too hard, too small, blemished, poor appearance, foreign matter, infested etc (Chtourou *et al.*, 2012). Date press cakes are being generated by the date palm agro industry and date processing industries (Chandrasekaran & Bahkali., 2013; Elsanhoty *et al.*, 2012).

Figure 4: Dry date (Boulal *et al.*, 2010)

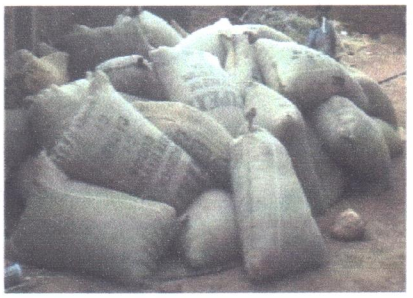

Figure 5: Dry date in the bag (Boulal *et al.*, 2010)

Packing operations generate a number of by-products for which valorization processes might be developed in order to improve the economy of the operation as a whole and to decrease disposal problems and costs and maximize return from industrial projects (Chtourou *et al.*, 2012; Jain *et al.*, 2011).The main by products (figure 6) especially date cull, date pits and date press

cakes have immense scop for value addition using bioprocess technologies (Chandrasekaran & Bahkali, 2013). The composition of each of this major by-product is mentioned in (table 5).

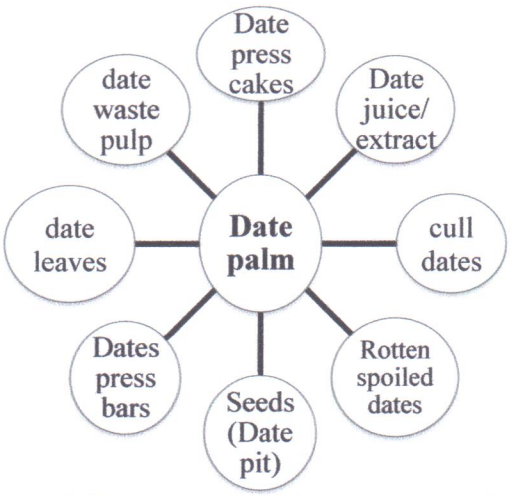

Figure 6: Date palm fruit by products and wastes.

Table 5: Essential components of the major date by products (Shahidi & Alasalvar, 2013; Chandrasekaran , 2012).

By products Composition (%)	Press cakes	Date seed	Date cull
Protein	3.62-5.23	2.29 -5.40	1 -2.5
Fate	1.40-2.20	5.02- 5.90	0.1 -0.5
Miosture	8.30 -10.59	3.14 -5.19	18±0.3
Ash	1.68 -2.46	0.89 -1.16	1.5±0.1
Carbohydrate	81.86- 83.33	83.14 -86.89	75 -85
Dietary fiber	25.39- 33.81	77.75- 80.15	62±1

Date by-products can be used as raw materials for the production of value-added products such as organic acids, exopolysaccharide, antibiotics, date flavored probiotic fermented dairy and bakery yeast etc (Jain *et al.*, 2011; Tang *et al.*, 2013). Some products are listed in (table 6). It does not require any special treatment like acid hydrolysis, steam explosion, or enzymatic treatment to release sugars in fermentable form (Tavakkoli *et al.*, 2012; Manickavasagan *et al.*, 2012).

II.3.2.3. valorization of date waste/by-product for probiotic biomass production

Large scale production of biomass necessitates the use of cheap substrates for economic production of the commodities by fermentation technologies. In this context few investigations have been reported on the use of date fruit by-products and wastes for the production of biomass such as probiotic lactobacilli and thermophilic dairy starters strain *Streptococcus thermophilus* (Chandrasekaran & Bahkali, 2013).

In the biotechnologies processes, Dates will serve mainly as carbon and energy sources for the microorganisms, in addition to other nutrients such as minerals and vitamins. In this respect, date can compare very well with other conventional substrates used in industrial fermentations such as molasses (Al-Eid *et al.*, 2010). Date palm (*P. dactylifera*) offers scope as a potentially convenient and inexpensive substitute for the glucose required by the lactobacilli during their growth and biomass production (Shrhavy *et al.*, 2012).Dates syrup and date pits were reported to have positive influence as nutrients for the cultivation of *Lactococcus lactis* and hence were suggested a suitable substrate for the cultivation of microorganisms (Elsanhoty *et al.*, 2012).

Table 6: products derived from date fruit by-products and wastes.

Substrate	Microorganism	Product	Reference
Date syrup	-*Aspergillus niger* J4	-citric acid	- Mostafa *et al.*, (2012)
	- *Aspergillus niger* ATCC 16404		- Acourene *et al.*, (2010)
	-*Streptomyces mobaraensis* ATCC 15003	-Bleomycin	- Radwan *et al.*, (2010)
Date seed	-	biodiesel	Amani *et al.*, (2012)
Date pomace	*Aspergillus niger* PC5	endopectinase	Bari *et al.*, (2010)
Date juice	- *Lactobacillus casei* subsp. *rhamnosus*	-lactic acid	-Chauhan *et al.*, (2006)
	- *Xanthomonas campestris NRRL B-1459*	-xanthan gum	-Salah *et al.*, (2010)
	-*Candida utilis.*	-Single cell protein	-Jain *et al.*, (2012)
	- *Corynebacterium glutamicum* 2262	-glutamic acid	-Mouffok *et al.*, (2010)
	- *Streptomyces albidoflavus and Streptomyces antibioticus*	-Vitamine B12	-Chandrasekaran *et al.*, (2013)
Date molasses	*Bacillus subtilis* B20	Biosurfactant	Al-Bahry *et al.*, (2012)
Dates wastes	*Candida guilliermondii* CGL-A10	Alpha amylase, biomass, citric acid, ethanol.	Acourene *et al.*, (2012)
Date powder	Lactobacillus casei 334	Probiotics Lactobacilli	Shahravy *et al.*, (2012)
Spoilage date palm	*Clostridium acetobutylicum* and *Bacillus subtilis*	acetone-butanol-ethanol	Abd-Alla and El-Enany, (2012)
Date extract	-*Aspergillus niger*	-Fungal protein	Manickavasagan *et al.*, (2012)
	-*X campestris PTCC 1473*	-Xanthan gum	-Farhadi *et al.*, (2012)
Date extract, date-coat sugar extract, date seed hydrolysat	*Saccharomyces cerevisiae*	Biomass production	Manickavasagan *et al.*, (2012)
Date waste pulp	*Zymomonas mobilis*	Bioethanol	Manickavasagan *et al.*, (2012)
Cull dates	*Candida kefir* CTM 30057	Ethanol	Chtourou *et al.*, (2012)

Shahravy *et al.*, 2012 used date powder for the first time as a low-cost main carbon source during the optimization of culture conditions for the economic production of a probiotic bacterium, *L. casei* ATCC 334. In this study the effect of eleven factors on bacterial growth was investigated using the Taguchi experimental design, and three factors including palm date powder (38 g/l), tryptone (30 g/l) and agitation rate (320 rpm) were found to be the most significant parameters by Response surface methodology of Box-Behnken.

Dates present a good vehicle to transport probiotics both for their micro-architecture and for the presence of nutrients. Date contain some micronutrients such as vitamins and minerals which might enhance the growth of microbial flora in probiotic dairy products. Addition of date syrup to probiotic yogurt enhanced the bifidobacterial count of the product (Jain *et al.*, 2011).

III. Materials and methods

III.1. Material

III.1.1. Bacterial strain and culture media

The bacterial strain used in this study was kindly provided by Dr. Tayeb Idoui. It was isolated from chicken gizzard at the Laboratory of Microbiology in the University of Jijel (Algeria) and it was identified by biochemical tests and by API gallery system (Biomerieux) for *Lactobacilli* identification as *Lactobacillus plantarum* G1. The strain was exhibited good probiotic properties (Riane &Majdoub, 2012).It was stored at -20°C in 20% (v/v) glycerol.

The bacterium G1 was activated by culturing it in Man Rogosa Sharpe (MRS) broth (20g glucose, 10g peptone, 10g meat extract, 5g yeast extract, 5g sodium acetate, 2g Bipotassic phosphate, 2g ammonium citrate, 0.2 magnesium sulfate, 0.05g manganese sulfate, 1.08 ml tween 80, pH 6.5) (Biokar Diagnostics. France) for 24h at 37°C. The broth was prepared with distilled water and sterilized by autoclaving at 121°C for 20 min. The pH was adjusted with NaOH 0.1N and HCl 1N. The purity of the strain was checked by streaking on MRS agar. Single colony was used and stained by Gram stain.

III.1.2. Raw material

Date wastes were collected from north of Algerian Sahara (City of Ghardaia), The morphologic observation indicate that there are several varieties of dates belongs to the category of dry / semi soft / soft dates, elongated / medium, large sized / medium, brown / light yellow / dark yellow / black color, with core / pitted with winkled appearance. It lost before and/or during the harvesting operation, picking, storage and conditioning processes. It usually used for animal feed.

Figure 7: Date wastes collected from Ghardaia

Reagents

- Sodium hydroxide (NaOH) 0,1N
- Hydrochloric acid (HCl) 1N
- Sodium hydroxide (NaOH) 1N
- Phenolphtalein indicator with 1%
- Phenol solution with 80 %
- Sulphuric acid concentrate
- Gallic acid
- Ethanol
- Folin ciocalteu
- Sodium of carbonate with 20%

Equipments

Equipments

- Vortex (IKA)
- Centrifuge (HETTICH ZENTRIFUGEN)
- pH meter (HANNA)
- Heat magnetic stirrer (Bunsen)
- Balance (Denver Instrument)
- Spectrophotometer (Shimadzu UV mini 1240)
- Whatman paper filters
- Pool bath shaker
- Muffle furnace
- Pool bath (Memmert)
- Ovens at 37°C, 30°C
- Refractometer (ATAGO-HAND-HELD Refractometer)
- Mineralisator.
- Analytical precision balance (KERN)
- Electric drying oven maintained at 103±2°C

III.2. Methods

III.2.1. Molecular identification of the *Lb. plantarum* G1 strain

Partial sequence of 16S rRNA was amplified by PCR technique, sequenced by automate sequencer after isolation and extraction of DNA. 16S rRNA was compared with the 16S rRNA of related microorganisms included in Genbank using BLAST sequence comparisons program. The Matrices of evolution distances were calculated and a phylogenetic tree was constructed by following the Neighbor-Joining's method using BioEdit program (Pancheniak *et al.*, 2012; Sharhavy *et al.*, 2012).

III.2.2. Preparation of date juice

For preparation of date juice, 160 g of washed and pitted dates were weighed and minced in distilled water and final volume was made up to 1200 ml.

Sugar extraction: The water–date mixture was heated at 80-85C° for 45 min with stirring, filtered through a cloth and sterilized at 121°C during 20 minutes. (Ould el hadj *et al.*, 2006; Acourene *et al.*, 2007; Banchabane *et al.*,2012), the clear juice was heated at 100 °C for 5 min in order to inhibit the enzyme activities. Finally, the juice was poured in flasks that will be cooled before taking place in the refrigerator. The juice alone was used as production medium or with the nutrient supplement of MRS medium, except the recommended concentration of sugar source (Elsanhoty *et al.*, 2012).

III.2.3. Date juice analysis

- **pH**

  The pH value was measured with a Hanna pH-meter.

- **Titratable acidity (TA)**

  It was carried out by measuring the citric acid that constitutes the major acid of date juice. This measure was achieved by neutralisation of the total free acids with a solution of NaOH. 40ml of juice has been titred by a solution of NaOH 1N, in presence of some drops of phenolphthalein indicator (Chaira *et al.*, 2007). To the zone of turn the colorful indicator, the volume of basis versed V1 has been noted and the acidity was expressed by the following equation:

$$A\,\% = \frac{(250\,.\,V1\,.100)}{(m\,.\,V\,.10)}0,06 = 150\frac{V1}{m\,.V}$$

With :

m: Mass of the sample (g).
V : Volume of filtrate taken for titration (ml).
$\mathbf{V_1}$: Volume of NaOH 1N (ml).
0.06: conversion Factor of titratable acidity as citric acid.

- **Totals Soluble solids**

The Brix (%) expresses the percentage of the soluble solids concentration in a sample (water solution), it represent the total of all dissolved solids in the water including sugars, salts, proteins, acids ...etc. Contents of total soluble solids (Brix°) were determined using a digital refractometer (Chaira *et al.*, 2007).

- **Dry matter**

The dry matter was determined after drying a sample in an isothermal oven at a temperature of 105° for 24h until a constant sample weight (Sánchez-Zapata *et al.*, 2011).
The water content was calculated by the following equation:

$$\mathbf{H\,\%} = \frac{(\mathbf{M1} - \mathbf{M2})}{\mathbf{P}} \times \mathbf{100}$$

With :

- **H %:** Humidity.
- $\mathbf{M_1}$**:** Mass of the capsule containing the fresh matter before steaming (g).
- $\mathbf{M_2}$: Mass of the capsule containing the fresh matter after steaming (g).
- **P:** Mass of the sample (g).

The result of dry matter was expressed in percentage (%):

$$\mathbf{Dry\ matter\% = 100 - H\ \%}$$

- **Ash**

The determination of the ash content is based on the destruction of all organic matter as a result of combustion in a muffle furnace at 550 °C for 3 h (g ash/100 g sample) (Kchaou *et al.*, 2013). Expression of results:

$$\mathbf{OM\,\%} = \frac{(\mathbf{M1} - \mathbf{M2})}{\mathbf{P}} \times \mathbf{100}$$

With:

- **OM**: organic matter.
- **M1**: Mass of capsule + The sample; M2: mass of the capsule + ash.
- **P**: Weight of the sample.

The ash content was calculated as follows:

$$\mathbf{Ash\ (\%) = 100 - MO\ \%}$$

- **Mineral matter content**

The sample was mineralized and placed in an acid solution. Then these solutions are atomized into a flame of acetylene and readings are taken at a wavelength of 285 nm by atomic absorption spectrophotometer (Acourene & Tama, 2001). The Cadmium, Chrome, Manganese, Lead, Zinc, Iron and Copper were determined after follow the following steps:

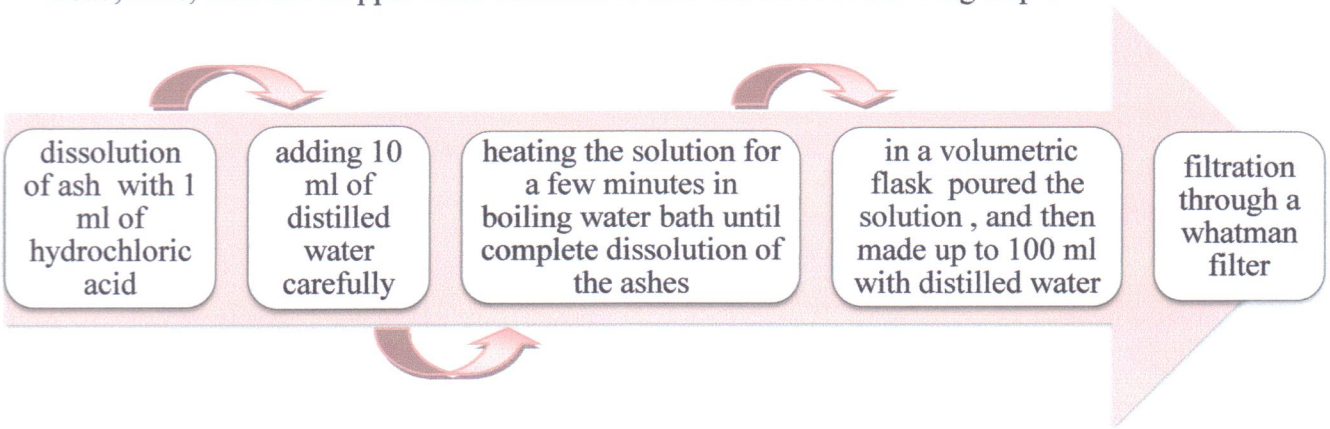

Figure 8: Diagram of sample preparation for the determination of the mineral content.

- **Protein assay**

Total nitrogen content was determined using the **Kjeldahl procedure** involving the destruction of the sample matrix and the conversion of nitrogenous matter to ammonium salts. The digestion was carried out with concentrated sulphuric acid at temperatures above its boiling point, using CuSO4/TiO2 as catalysts. The ammonium salt was then converted to ammonia by reaction with excess sodium hydroxide, the ammonia was steam-distilled off and trapped in a boric acid solution. The ammonia concentration of the distillate was determined by titration with a standardized acid solution, and its value expressed in NH_3–N using the appropriate calculations (Chtourou *et al.*, 2012). The total nitrogen content was determined by the following formula:

$$N\% = \frac{14(V-V').0,05}{P} \times 100$$

With:

V: Quantity of sulfuric acid read after titration (ml) with sulfuric acid normality of 0.05 N

V': Quantity of sulfuric acid read after titration (ml) with sulfuric in blank sample.

P: Weight of sample (g).

The **protein content** is calculated by multiplying the total nitrogen content N (%) by the factor **6.25** after follows the following stages:

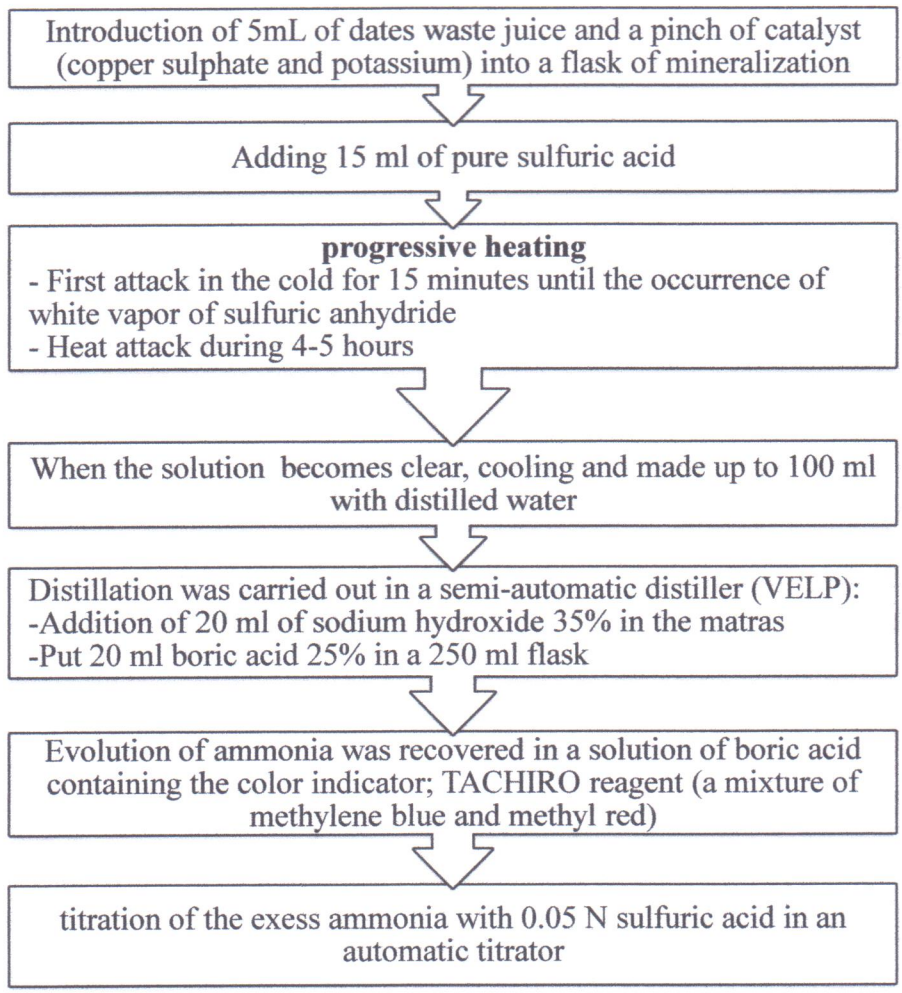

Figure 9: Stages of Kjeldahl procedure for the determination of total nitrogen content.

- **Total sugars content**

The technique used is that described by **Dubois *et al.*, (1956)**. In this technique the total sugars are first extracted with distilled water in the presence of concentrated sulfuric acid, the monosaccharides are dehydrated in compounds of the of family furfuriques derivatives. These products are condensed with phenol to give yellow-orange complex. The appearance of these complexes is monitored by measuring the increase in optical density at 490 nm. For the assay, a standard curve was previously prepared from 0.01% glucose solution. 0.05 ml of 80% phenol solution and 3 ml of concentrated sulfuric acid were added to each tube of the standard curve and the extract, followed by a slow and gentle agitation. The reaction mixture was left for 10 minutes at a temperature of 25-30°C (appearance of yellow-red color). The reaction was stopped by a stream of cold water and the optical densities were measured (annex I).

- **Total phenolic content (TPC)**

The TPC analysis was based on the methods described by **Juntachote *et al.*, (2006)** using a UV-visible spectrophotometer (Shimadzu) and Folin ciocalteau reagents. The optical density of the blue-colored samples was measured and was read in triplicate at 760 nm. The calibration curve was constructed with gallic acid and the total phenolic contents were expressed as mg equivalent gallic acid (GAE)/100g dry matter basis. Two hundred mg of gallic acid was dissolved in 100 ml of ethanol; a solution (S) with a concentration of 2mg/ml was prepared and diluted 9 times.

Date extract was diluted also 9 times before use. Approximately 0.5 of diluted samples was added into test tubes followed by 0.5 ml of Folin-Ciocalteu's reagent and 0.5 ml of sodium carbonate. The blank sample was prepared by replacing 0.5 ml of sample with 0.5 ml of distilled water. The test tubes were vortexed for 10 s and allowed to stand in the dark environment at room temperature for 1h. Absorbance was measured against the blank sample at 760 nm using UV light spectrophotometer. A calibration curve of gallic acid was plotted by plotting absorbance vs concentrations of gallic acid (mg/l) (annex II).

III.2.4. Optimizing the production of probiotic biomass in the presence of date extract

The objectif is to study some factors that affect the production of biomass in order to enhance it. In this order we used the one variable at a time method followed by Plackett-Burman design.

III.2.4.1. Inoculum preparation

For inoculum preparation, 1mL from reactivated culture was taken and used to inoculate 100 ml Erlenmeyer flask containing 20 ml of MRS broth. The inoculated flask was incubated at 37°C for16h.

III.2.4.2. Biomass estimation

The biomass concentrations (dry weight g/l) were determined using a calibration curve. The calibration curve was calculated using dilutions of a biomass suspension with known optical density (Rodrigues *et al.*, 2006). An Erlenmeyer flask containing 50ml of MRS broth was inoculated with 5ml of bacterial culture and was incubated at 37°C for 16h.

A fixed volume of the dilutions (10ml) with optical density measured by the UV-Vis spectrophotometer and was centrifuged at 6000× g for 20 min. The pellet was recovered with 100µl of distilled water, filtered through a whatman papers filters and left to dry at 105◦C (Panchiniak *et al.*, 2012). All the filters were weighed before filtration and after drying. Thus, a relationship between biomass concentration (g/l) and optical density (600 nm) can be determined.

III.2.4.3. The effect of different concentrations of date juice sugars on biomass production

Different concentrations of date juice were added to 100ml Erlenmeyer flasks containing 20ml of MRS broth without glucose (1%, 2%, 4%, 8%, 10%, 12%, 14%). The flasks were inoculated with 5% of a 16h bacterial culture and were incubated at 37°C for 48h. 20 ml of MRS broth with glucose inoculated and incubated in the same conditions was taken as a control. The optical density was measured at 600 nm after 24h and 48h.

III.2.4.4. The effect of inoculum size on biomass production

One hundred milliliter Erlenmeyer flasks containing 20 ml of MRS broth without glucose and 12% of date extract were inoculated with different concentration inoculums size, respectively (1%, 3%, 5%, 7%, 9%, 10%). The flasks were incubated at 37°C for 24h. The optical densities were measured at 600 nm by UV-Vis spectrophotometer and the inoculum size which gives the higher biomass production was appointed.

III.2.4.5. Plackett-Burman design

For screening purpose, various medium components have been evaluated using Plackett-Burman statistical design, which is a fraction of two-level factorial design and allows the investigation of n-1 variables in at least n experiments. In the present study, Plackett-Burman design was used to screen the important medium components with respect to their main effects and not the interaction effects between various medium constituents.

Including date juice, a total of seven components [variables, k= 7] were selected for the study and each variable represented at two levels, high concentration (+) and low concentration (−) (Table 7).The number of positive signs and negative signs per trial are (k + 1)/2 and (k - 1)/2, respectively. Each column should contain equal number of positive and negative signs. Thus, each row represents a trial run and each column represents an independent (assigned) or dummy (unassigned) variable which is used to estimate experimental errors in data analysis. The significant factors for biomass production were based on the first-order model as given in equation 1.

$$Y = \beta_0 + \Sigma \beta i\, xi \ldots\ldots (1)$$

Where Y is the response value (biomass production), β_0 is the model intercept and β_i is the linear coefficient, X_i represents the level of the independent variable.

A total of 8 sets of experiments were done in 100 ml Erlenmeyer flask containing 20ml of each medium in order to determine the significant factors affecting production of biomass. The Erlenmeyer flasks were inoculated with a 16h bacterial culture and incubated for 24h. The biomass concentration was determined.

All experiments were carried out in duplicate and the averages of biomass concentration were taken as responses. The variable with confidence levels above 95% is considered the most significant factor that effects the biomass production of *Lb. plantarum* G1. The Pareto plot best demonstrate results of Plackett-Burman design that illustrates the absolute relative significance of variables independent on their nature.

Table 7: Media components and test levels for Plackett–Burman experiment.

variable	Variable code	Low level (-1)	High level (+1)
Date extract (%)	X1	10	14
Yeast extract (g/l)	X2	5	10
Peptone (g/l)	X3	10	20
Tween (g/l)	X4	1	6
Ammonium citrate (g/l)	X5	0.5	2
T°C	X6	30	37
pH	X7	5.5	6.5

III.2.4.5.1. Data analysis

The data on probiotic biomass yield was subjected to statistical analysis using MICROSOFT EXCEL 2003 software to estimate t-value, p-value and confidence level. The student's t-test for any individual effect allows an evaluation of the probability of finding the observed effect purely by chance. The Confidence level is an expression of the P-value in percent.

III.2.4.6. antimicrobial activity of metabolites produced by *Lactobacillus* isolates

Antimicrobial effects of Lactobacillus isolate against pathogenic strains were determined by the agar diffusion method. The same model of PB design (Table 7) was used to estimate the effect of different factors including date extract on the production of antimicrobial agents from *Lb. plantarum* G1. *Lb. plantarum* G1 was grown in eight modified MRS broth and centrifuged at 6000×g for 30 min. The supernatant, after being sterilized by filtration through a 0.45 µm Millipore filter (Pancheniak *et al.*, 2012), was tested against pathogenic strain: *Salmonella* sp. This indicator strain was grown in nutrient broth at 37°C for 24 h.

The assays were performed in duplicate. 110µl of indicator bacteria suspension was inoculated into 20 ml of Muller Hinton agar cooled to 45°C. The mixture was pooled in Petri dishes and incubated for 4h at 37°C. Eight wells of 6 mm diameter were cut into these agar plates using a sterile tip and 50 µL of each cell free supernatants (CFS) collected from 24 h bacterial culture were poured into wells. The plates were incubated aerobically overnight at 37°C and the inhibitory activity was examined for the clear inhibition zone surrounding each agar well (Kermanshahi & peymanfer, 2011). The diameter of inhibition zone was measured to indicate the magnitude of inhibitory activity.

III.2.4.7. Estimation of Lactic acid

The amount of lactic acid in each fermentation broth of Plackett-Burman design was determined by transferring 3 ml of culture broth supernatant of LAB isolate into 10 ml flask. 120 µl of phenolphthalein indicator was added into the flask. This was titrated with 0.25 M NaOH under Heat magnetic stirrer (Bunsen) for the appearance of pink color. The titratable acidity was calculated as lactic acid % W/V. Each milliliter of 1 N NaOH is equivalent to 90.08 mg of lactic acid. The titratable acidity was then calculated. Data of PB design was analysed as described above (Sheeladevi *et al.*, 2011).

III.2.4.8. pre-optimization experiment

It was realized in order to evaluate the results of Plackett- Burman Design. In this step the significant variables obtained from Plackett-Burman design analysis that affect biomass production were put in high value (+1) except peptone which was changed between low value (-1) and high value (+1). The others parameters which have a negative effect were regard with their low level (-1) except ammonium citrate which was changed (Table 8).

Table 8: Media components and test levels for pre-optimization test.

Medium Composition	Pre-optimized medium I	Pre-optimized medium II	Pre-optimized medium III	Pre-optimized medium VI
Date juice (%)	+	+	+	+
Yeast extract (g/l)	+	+	+	+
Peptone (g/l)	-	-	+	-
Tween-80 (g/l)	-	-	-	-
Ammonium citrate (g/l)	-	-	+	+
T°C	-	-	-	-
pH	-	-	-	-

IV. Results and discussion

IV.1. Molecular characterization

The isolated strain *Lb. plantarum* G1 was molecularly identified and the partial sequence (Figure 10) obtained after the isolation of 16S rRNA gene was analyzed using the BLAST routine of the Genbank. The sequences obtained from BLAST analysis of 16S rRNA and phylogenetic tree are presented in Figures (11) and (12). The Results indicated that the isolate under study was 99 % closer to *Lb. plantarum*. Moreover, the nucleotide sequence (843 base pairs) was deposited in the GenBank sequence database and given the accession number KC965107. The phylogenetic analysis confirmed a closer phylogenetic proximity of the G1 sample with the *Lb. plantarum* species.

PCR-sequencing based technique is based on the fact that 16S rDNA gene is actually species specific. Thereby the 16S rRNA gene has been considered as the gold standard and is extensively used to derive useful taxonomic information, particularly with regard to identification at the species level (Kumar *et al.*, 2011). The 16S rDNA contains conserved sequences that can be used to infer natural relationships between distantly related species and variable regions that can be used to separate closely related ones (Chen *et al.*, 2000; Kumar *et al.*, 2011).

16S rDNA analysis has been demonstrated to be a powerful tool to investigate the biological diversity. These methods have several advantages: The structure of DNA remains stable with physiological stages, its composition does not depend on culture conditions and it has regions with different degrees of variability that allow species specific detection or the detection of wider taxonomic groups (Hosseini *et al.*,2012). Accurate genotypic identification is extremely important to link the strain with correspond species and specific health effect (Kumar *et al.*, 2011).

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Gcagtcgacgactctggtattgattggtgcttgcatcatgatttacatttgagtgagtggcgaactggtgagtaacacgtgggaaa
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```

Figure 10: Partial sequence of 16S rRNA gene of *Lb. plantarum* G1.

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```
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cgtaaacgatgaatgctaagtgttggagggtttccgccttcagtgctgcagctaacgcattaagcattc
```

Figure 10: Partial sequence of 16S rRNA gene of *Lb. plantarum* G1.

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Lactobacillus plantarum strain CHE04 16S ribosomal RNA gene, partial sequence	1546	1546	99%	0.0	99%	EF426285.1
Lactobacillus plantarum strain GRctBL101 16S ribosomal RNA gene, partial sequence	1544	1544	99%	0.0	99%	KC351898.1
Lactobacillus plantarum gene for 16S ribosomal RNA, partial sequence, strain: qz145-1	1544	1544	99%	0.0	99%	AB761311.1
Lactobacillus plantarum 16S ribosomal RNA gene, partial sequence	1544	1544	99%	0.0	99%	JX423550.1
Lactobacillus plantarum strain R30-1 16S ribosomal RNA gene, partial sequence	1543	1543	100%	0.0	99%	KC479667.1
Lactobacillus plantarum strain LN-9 16S ribosomal RNA gene, partial sequence	1543	1543	100%	0.0	99%	JX413792.1
Lactobacillus plantarum strain MNC29 16S ribosomal RNA gene, partial sequence	1543	1543	100%	0.0	99%	JQ754460.1
Lactobacillus plantarum strain YML007 16S ribosomal RNA gene, partial sequence	1543	1543	100%	0.0	99%	JN853603.1
Lactobacillus plantarum strain JJ 55 16S ribosomal RNA gene, partial sequence	1543	1543	100%	0.0	99%	JN573603.1
Lactobacillus plantarum subsp. plantarum strain JJ 60 16S ribosomal RNA gene, partial sequence	1543	1543	100%	0.0	99%	JN573602.1
Lactobacillus plantarum gene for 16S rRNA, partial sequence, strain: SCP57	1543	1543	100%	0.0	99%	AB617651.1
Lactobacillus plantarum strain LP-01 16S ribosomal RNA gene, partial sequence	1543	1543	100%	0.0	99%	HQ441200.1
Lactobacillus plantarum strain ChR-I-str20 16S ribosomal RNA gene, partial sequence	1543	1543	100%	0.0	99%	HM462422.1
Lactobacillus plantarum strain DSPV 354T 16S ribosomal RNA gene, partial sequence	1543	1543	100%	0.0	99%	FJ751793.1
Lactobacillus sp. 0-C-2 16S ribosomal RNA gene, partial sequence	1543	1543	100%	0.0	99%	GQ359860.1

Figure11: Data analysis with BLAST search program.

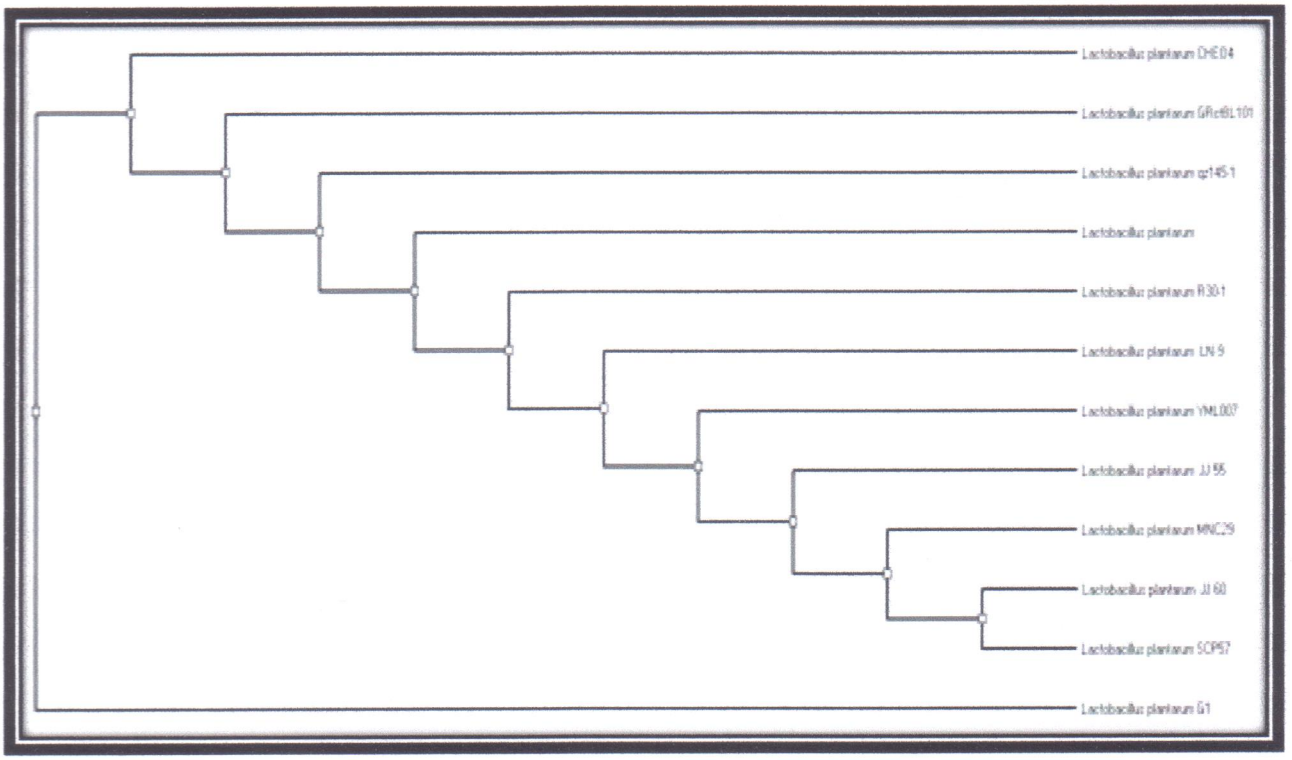

Figure 12: Phylogenetic relation of *Lb. plantarum* G1 strain sequence with 16S rDNA of the highest 11 similar bacteria. The dendogram was generated by the neighbour-joining method using Bio-Edit software.

Lactobacillus plantarum strain G1 16S ribosomal RNA gene, partial sequence

GenBank: KC965107.1

FASTA Graphics

Go to:

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  ORGANISM  Lactobacillus plantarum
            Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae;
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REFERENCE   1  (bases 1 to 843)
  AUTHORS   Idoui,T., Ouled-Haddar,H. and Sifour,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (24-APR-2013) Department of Cell and Molecular Biology,
            University of Jijel, Ouled Aissa, Jijel, Jijel 18000, Algeria
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781 cgatgaatgc taagtgttgg agggtttccg ccttcagtgc tgcagctaac gcattaagca
841 ttc
```

Figure 13: Registration sheet concerning data of *Lb. plantarum* G1.

Lactobacillus plantarum strain G1 16S ribosomal RNA gene, partial sequence

GenBank: KC965107.1

FASTA Graphics

Go to:

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ACCESSION   KC965107
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KEYWORDS    .
SOURCE      Lactobacillus plantarum
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            Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae;
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REFERENCE   1  (bases 1 to 843)
  AUTHORS   Idoui,T., Ouled-Haddar,H. and Sifour,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (24-APR-2013) Department of Cell and Molecular Biology,
            University of Jijel, Ouled Aissa, Jijel, Jijel 18000, Algeria
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721 tgaggctcga aagtatgggt agcaaacagg attagatacc ctggtagtcc ataccgtaaa
781 cgatgaatgc taagtgttgg agggtttccg ccttcagtgc tgcagctaac gcattaagca
841 ttc
```

Figure 13: Registration sheet concerning data of *Lb. plantarum* G1.

IV.2. General Characteristics of the filtered date waste juice

The different categories of date wastes used in this study have different morphological and organoleptic characteristics. These differences are notable between the soft, semi-soft and dry variety, especially from the viewpoint of color, consistency, texture and in the same relative core /date. The nucleus/date show that the semi-soft variety is thick compared to the soft and semi-soft varieties. The date has a dry chalky and hard texture, in contrast, soft and semi-soft date, have fibrous textures. Morphological properties of date fruit affect the physicochemical characteristics of products prepared from date ex.date juice (Sayah & Ould el hadj, 2010).

After filtration and sterilization, the obtained date juice was yellow liquid, dark, slightly, viscous, cloudy. A little foam formed on the surface when the juice was mixed. The resulting foams are indicative of the presence of surfactants and the cloudiness of the juice is due to impurities in suspension, namely pectins and proteins (Benchabane *et al.*, 2012). For organoleptics characteristics, date juice has a very strong odor of date.

Figure 14: date juice prepared from date wastes

IV.3. Date juice analysis

Any process of fermentation is related to the quality of the used culture medium. Therefore, the determination of the biochemical composition of date juice is required. The chemical composition of dates can vary, depending on cultivar, soil conditions, and agronomic practices, as well as the ripening stage. Date fruits pass through several separate stages of maturity, traditionally described by changes in color, texture and taste/flavor (El Arem *et al.*, 2011). Consequently, the chemical composition of date juice is changed under the effect of these factors. The physicochemical characteristics of date waste juice are summarized in Table (9).

Table 9: Physicochemical parameters of date juice

Parameters	Date waste juice
pH	6.35
Titratable acidity (%)	0.31
TSS°Brix	61
Dry matter	2.6g =9.37%
Humidity (%)	90.63
Ash (%)	1.11
Total sugars	35g/l=6.6g/100g FM
Total nitrogen (%)	0.053
Protein (g/100g)	0.331
Total phenolic (mg/ml)	14.53
Cadmium mg/100 ml fresh matter	0.011
Chromium mg/100 ml fresh matter	4.72
Manganese mg/100 ml fresh matter	0.82
Lead mg/100 ml fresh matter	0,35
Zinc mg/100 ml fresh matter	1.57
Iron mg/100 ml fresh matter	2.53
Copper mg/100 ml fresh matter	0.99

- **pH and titratable acidity (TA)**

From the obtained results (Table 9), it observed that the pH value for the tested date juice was 6.2 and its TA value was found to be 0.31% (as citric acid, on wet weight basis). Our finding is compared to previously reported results from literature. Benchabane *et al.*, (2012) found that the pH value of date juice prepared from Ghars variety collected in the last stage of maturity was similar to our result (6.2) and higher than that obtained by Acourene *et al.*, (2001) and Ould el hadj *et al.*, (2012), which were 5.9, 5.82, for date juice prepared from Tinissine (Touggourt) and Techerwit varieties, respectively.

The pH is a parameter determining the aptitude for the conservation of food. It is one of the main obstacles that microbial flora must take to ensure its proliferation. The lactic acid bacteria prefer neutral mediums, usually between pH 7 and 7.9 with the most tolerance to variations between 6 and 9 (Sayah & Ould el hadj, 2010).

Concerning TA or percentage of the citric acid; this last is in general an intermediary of the metabolic processes. It influences the growth of the micro-organisms and affects the quality of conservation of the products. According to Acourene *et al*, (2007), acidity is dependant on the conditions of fertilization and irrigation on the palm trees.

- **Dry matter and moisture**

Moisture was the predominant component in all date varieties, juices, and their by-products. The results showed that the date juice had 2.6g (9.37%) of dry matter and 90.63% of moisture. Our findings are comparable with other date juice prepared from different varieties. Ould el hadj *et al.*, (2006) reported that the dry matter and humidity were 11.2 ± 1.07%,

Table 9: Physicochemical parameters of date juice

Parameters	Date waste juice
pH	6.35
Titratable acidity (%)	0.31
TSS°Brix	61
Dry matter	2.6g =9.37%
Humidity (%)	90.63
Ash (%)	1.11
Total sugars	35g/l=6.6g/100g FM
Total nitrogen (%)	0.053
Protein (g/100g)	0.331
Total phenolic (mg/ml)	14.53
Cadmium mg/100 ml fresh matter	0.011
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88.8±2.57 %, respectively. Benchabane *et al.*, (2012) found that dry matter was equals to 10.57% and the humidity was 89.43%.

- **Total Soluble Solids (TSS)**

As illustrated in Table (9), the total soluble solids (Brix°) for date juice was 61°, this result was compared with those reported by Acourene *et al.*, (2001) which was found that the total soluble solids for tested date juice from Deglet Nour, Tantboucht and Tinissine varieties were 21°, 22.5°, 23.5°, respectively. For food, the date juice was concentrated by evaporation to obtain a TSS of 76°. This date juice was found to be stable over 6 months, the use of pectinase/cellulase facilitated the highest recovery of total soluble solids (65.6 – 70.7%) compared with control (50.5 – 56.30%) (Chandrasekaran *et al.*, 2013). TSS depends to the variety and the methods of extraction of sugar.

- **Ash**

The ash content represents the total amount of minerals present in the sample analyzed. It is expressed in % of dry matter. The ash content of our date juice was 1.11% of dry matter. The obtained values is in the same line with that reported by Acourene *et al.*, (2001) for juice extracted from Deglet Nour and slightly lower than juice from Tinissine (1.34) and Tantboucht (1.49%) varieties. However, Ould el hadj *et al.*,(2006) reported a concentration of 1.78±0.05 for date juice prepared from date waste (H'Chef). The difference in ash content may be due to the difference in climatic and storage conditions (Anjum *et al.*, 2012).

- **Protein content**

As illustrated in Table 9, date juice contained 0.331 g/100g (% fresh matter FM) of protein, This result is in accordance with those found by Ould el hadj *et al.*, (2012) in juice of Techerwit variety and lower than in Degla Beida (0.53%), Hamraya (0.67%) varieties. Acourene *et al.*, (2001) reported that Deglet Nour, Tantboucht and Tinissine juice contained 0.24 %, 1.05%, 0.80% of protein, respectively. Although protein content is low, previous works have determined that it contains 23 types of amino acids, some of which are not present in most popular fruits (Sanchaez-Zapata *et al.*, 2011).

- **Total sugars content**

The sugar percentage and type change according to date varieties, consistence and maturation state (Sanchaez-Zapata *et al.*, 2011). According to the date juice and on a fresh weight basis, the total sugars content was equal to 6.6%. This value was less than that obtained by Ould el hajd *et al.*, (2006) (16.64±1.08). Acourene *et al.*, (2007) obtained a date juice with content of total sugars (22.61% of FM) from offal's of Deglet- nour. In another study, Ouled el hajd *et al.*, (2012) extracted only 11.61± 0.26% and 11.00±0.21 from Degla Beida and Hamraya variety. This result confirmed that the sugar content depend on the variety.

- **Total phenolic**

The determination of total phenolics gives us an overall estimate of the amount of different classes of phenolic compounds in date extracts. As shown in Table (9), total phenolic content in date juice was 14.53mg/ml. Mansouri *et al.*, (2005) analyzed mature date varieties from valley of Ghardaïa and they showed that date contains overall a low total phenolic rate.

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The concentrations and ratio of polyphenols depend on the stage of fruit picking, variety of date, location and soil conditions, geographic origin, fertilizer, storage conditions, cultural methods, process and stabilisation conditions, use of different analytical methods and use of different phenolic acid standards. These phytochemicals components also add to the nutritional and organoleptic properties of the fruit (Abbas *et al.*, 2008; Anjum *et al.*, 2012).

Fresh dates fruits are an excellent source of natural antioxidants and some of them even outperform the synthetic antioxidants, and are safer also from the health point of view (Anjum *et al.*, 2012). Phenolic compounds have been reported to present multiple biological effects, including reducing the risk of heart disease, cancer, cataracts, etc. regeneration of certain antioxidants such as vitamin E and formulation of fruit colour (Scalbert *et al.*,2002).

Antioxidant defences of polyphenols are paramount importance to act to protect nerve tissue against oxidative damage such as those found in disease Alzaheimer (Henk *et al.*, 2003; Ganbi, 2012). Moreover, it has been reported that phenolic compounds play a role in the defense mechanisms against microbial invasion and UV rays they exert an inhibitory action on numerous bacteria, fungi and even viruses (Rodriguez *et al.*, 2007).

- **Mineral matter content**

The date contained significant amount of important minerals (Table 9), results showed that Chrome was the predominant mineral in our date juice (4.72mg/100ml FM), followed by Iron (2.53mg/100 ml FM), Zinc (1.57 mg/100 ml FM), Copper (0.99 mg/100 ml FM), Manganese (0.82 mg/100 ml fresh matter), Lead (0,35 mg/100 ml fresh matter) and Cadmium with low content (0.011 mg/100 ml FM). Our results were comparable with those reported by Acourene *et al.*, (2007). Iron content was similar with that obtained in Deglet Nour juice and smaller than that found in Tantboucht juice (5.86 mg/100 ml FM). Manganese, Copper and Zinc content were higher than that obtained in Deglet Nour juice (0.07, 0.07 and 0.25 mg/100 ml FM). For the role of minerals we can take the Zinc as example which plays an antioxidant role and it can prevent the oxidation of proteins (Kchaou *et al.*, 2013).

IV.4.Optimizing the production of probiotic biomass in the presence of date extract

The optimization process was carried out using date juice as source of carbon supplemented with other components of MRS for *Lb. plantarum* G1growth.

IV.4.1. Biomass estimation

Biomass was expressed as dry cell mass. The OD of culture was converted to dry cell mass through a linear correlation standard curve demonstrated bellow. The estimation of biomass concentration with the calibration curve of biomass dry weight and optical density is a simple method whereas the others techniques such as counting the colony forming units (CFU) which have time consuming and in case it is difficult to determine the number of colonies. The calibration curve technique is widely used, but it remains an estimation method.

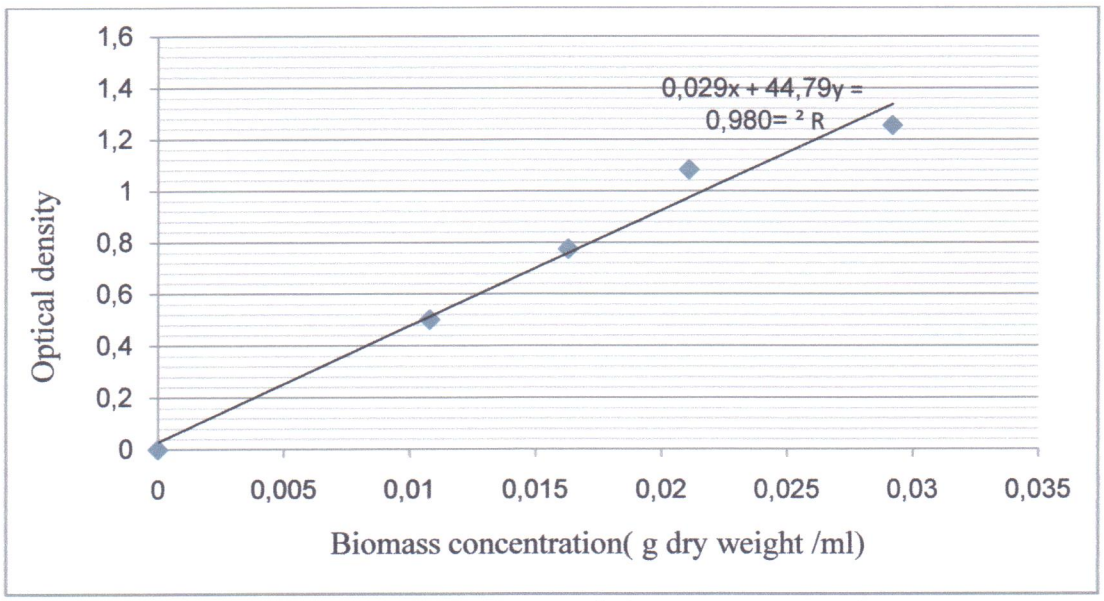

Figure 15: calibration curve of biomass concentration

IV.4.2.Date juice as substrate for lactic acid bacteria growth

According to the obtained results, the moisture and carbohydrate were predominant component in date juice, followed by dry matter and ash, along with small amounts of total phenolics, protein and mineral matter. With regard to the amounts of total sugars content, the date juice may be a potentially convenient and inexpensive substitute for the glucose required by the Lactobacilli.

In order to evaluate the date juice as medium for *Lb. plantarum* G1 growth and in the same time we had realized a comparison between the biomass production in MRS standard medium and in date juice alone, two tubes containing 10 ml of MRS broth, date juice respectively. The tubes were inoculated and incubated for 24h at 37°C. After measured the optical densities, it converts to biomass concentration (g/l) and the obtained results are presents in the table (10)

Table 10: Biomass production and metabolites production in MRS medium and date juice alone.

Medium	Optical density	Biomass concentration (g/l)	Antimicrobial activity (cm)	Acidity (g/l)
MRS broth	1.053	22.85	1.2	24
Date juice	0.710	15.19	1	9.75

The results show that the date juice is favorable to the growth of bacterial strain because it contains a high amount of sugars easily assimilated by *Lb. plantarum*. The biomass production in MRS medium is higher than that obtained in date juice alone. The antimicrobial activity is equal in the two medium and the acidity in MRS medium is higher when compared with date juice acidity.

The results of date juice may be interpreted by the low protein and mineral matter content in date which is insufficient for Lactobacilli growth whereas MRS broth is a rich medium. Date

juice has a greater feasibility to be utilized as a substrate for the production of some economically important microbial products. Any material that contains this nutrient could serve as a possible alternative substrate for microbial fermentation (Manickavasagan *et al.*, 2012).

According to Ould el hadj *et al.*, (2012) date extract constitute a better fermentation medium for microorganism growth. It is rich in minerals matter including calcium, potassium and phosphorus but poor in magnesium, thereby the medium need enrichment. Acourene *et al.*, (2007) reported that the best culture medium in industrial biochemistry is one that provides the best production in the shortest time and whose cost is as low as possible. According to Nancib *et al.*, (2001) date alone is a poor nitrogen source and salts for *Lb. rhamnosus* growth and lactic acid production. Thereby, different supplementations, such as yeast extract, MgSO4, K2HPO4, KH2PO4 and Tween-80 have been proposed. In other study, Elsanhoty *et al.*, (2012) used date juice as a medium for carotenoid production *Lb. plantarum* strains, they observed that the date juice when used alone produce a significant recovery. But when supplemented with salts and organic nitrogen of MRS medium the yield was increased.

IV.4.3.The effect of different concentrations of date juice sugars on biomass production

Different concentrations of total sugars content in date juice were tested (Table 11). The results show that the date juice is favorable to the growth of bacterial strain. Indeed, it was found that the biomass increases after addition of total sugars content in date juice reaching maximum yield (25.38g/l) when the culture medium contains 12% of total sugars, after this value the biomass concentration decreases, nevertheless the total sugar content increases.

Table 11: the biomass concentration in MRS medium and MRS without glucose supplemented with different concentration of date juice sugars.

date juice Concentration (%)	**0 (MRS with glucose)**	**1**	**2**	**4**	**8**	**10**	**12**	**14**
OD after 24h	1.160	0.693	0.961	1.062	1.109	1.124	1.166	1.124
Biomass concentration(g/l) after 24h	25.2	14.8	20.8	23.5	24.1	24.4	25.38	24.4
OD after 48h	1.209	0.650	0.702	1.013	0.973	1.109	1.084	1.044
Biomass concentration(g/l) after 48h	26.3	13.86	15.02	21.96	21.07	24.1	23.5	22.65

According to the results, the biomass concentration obtained in broth content12% of date juice which replace glucose and supplemented with salts, organic nitrogen sources was very significant in comparison with the biomass concentration obtained with MRS standard medium.

After 48 h the biomass concentration decreases with all total sugar content concentration tested. This decreasing may be due to the bacterial strain lyses before the pre-incubation.

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After 48 h the biomass concentration decreases with all total sugar content concentration tested. This decreasing may be due to the bacterial strain lyses before the pre-incubation.

With regard to sugars content, date juice provide a good source of rapid energy because most of the carbohydrates are in the form of fructose and glucose, which are easily assimilated by *Lb. plantarum* G1 their growth and metabolites production.

IV.4.4.The effect of inoculum size on biomass production

The biomass concentration increases with inoculum size reaching the higher biomass concentration of 28.7 obtained with 5% inoculum size. After this value the biomass decreases despite the increasing in inoculum size. This decreasing due to the competition on the nutrients.

Table 12: biomass concentration in MRS modified with different concentration of inoculum size.

Inoculum size	1%	3%	5%	7%	9%	10%
OD after 24h	1.215	1.195	1.315	1.307	1.201	1.139
Biomass concentration g/l	26.4	26.02	28.7	28.52	26.16	24.77

IV.4.5.Plackett-Burman Design

IV.4.5.1. Evaluation the effect of nutrient components on biomass production

Eight mediums were examined using Plackett-Burman statistical experimental design to select the factors affecting biomass production in order to improving it and reduce the cost.

The main effect of each variable upon biomass production were calculated and presented graphically in Figure 16. The data in Table 13 showed a variation from 16.71 to 24.21 g/l of biomass production. This variation shows the strong influence of medium components on biomass production and reflects the importance of medium optimization to attain higher productivity.

Table 13: Plackett-Burman design for biomass production by *Lactobacillus plantarum* G1

Trials	X1 (Date juice)	X2 (yeast extract)	X3 (peptone)	X4 (Tween)	X5 (Ammonim citrate)	X6 (T°C)	X7 (pH)	Biomass g/l
1	10(-1)	10(+1)	20(+1)	1(-1)	2(+1)	37(+1)	5.5(-1)	22.96
2	10(-1)	5(-1)	20(+1)	6(+1)	0.5(-1)	37(+1)	6.5(+1)	16.71
3	14(+1)	5(-1)	10(-1)	6(+1)	2(+1)	30(-1)	6.5(+1)	19.62
4	14(+1)	10(+1)	10(-1)	1(-1)	2(+1)	37(+1)	5.5(-1)	23.97
5	10(-1)	10(+1)	20(+1)	1(-1)	0.5(-1)	37(+1)	6.5(+1)	24.21
6	14(+1)	5(-1)	20(+1)	6(+1)	0.5(-1)	30(-1)	6.5(+1)	21.29
7	14(+1)	10(+1)	10(-1)	6(+1)	2(+1)	30(-1)	5.5(-1)	23.75
8	10(-1)	5(-1)	10(-1)	1(-1)	0.5(-1)	30(-1)	5.5(-1)	**20.71**

On the basis of the analysis of the regression coefficients of the seven variables after 24 hours of incubation: yeast extract had the major positive influence on biomass production, followed by date juice and peptone, whereas Tween 80, Temperature, pH had a significant negative influence. When the sign of the effect of the tested variable is positive, the biomass production is greater at a high level of the parameter, and when negative, the biomass production is greater at a low level of the parameter.

Thereby, if the effect was positive, a higher concentration than the indicated high value (+) concentration was required during further optimization studies and if the effect was negative, it indicated that the component was effective in biomass production but the amount required was lower than the indicated as low (−) concentration in Plackett–Burman experiment (Kiruthika *et al.*, 2011, Chauhan *et al.*, 2007).

On the basis of the calculated t-values and confidence level (%) in Table 14, the variables with highest confidence levels were considered as most significant on probiotic biomass production. In this study date juice, yeast extract and peptone were found to be the most significant variables affecting biomass production.

Table 14: The regression analysis of the effect of each variable along with the coefficient level, t and P value.

Variables	Coefficient	*t-value*	*P-value*
Date juice (%)	13.19	4,30324984	0,1453594
Yeast extract (g/l)	27.75	9,0509668	0,07005309
Peptone (g/l)	6.93	2,2627417	0,26491818
Tween- 80 (g/l)	-16.54	-5,39421459	0,11669421
Ammonuim citrate (g/l)	-5.75	-1,81827458	0,32010622
T°C	-15.17	-4,94974747	0,12690837
pH	-14.8	0,86813437	-1,37651501

Figure 17 showed the ranking of factor estimates in a Pareto chart. The Pareto chart displayed the magnitude of each factor estimate and offers a convenient way to view the results obtained by Plackett-Burman design (Rajendran *et al.*, 2007).

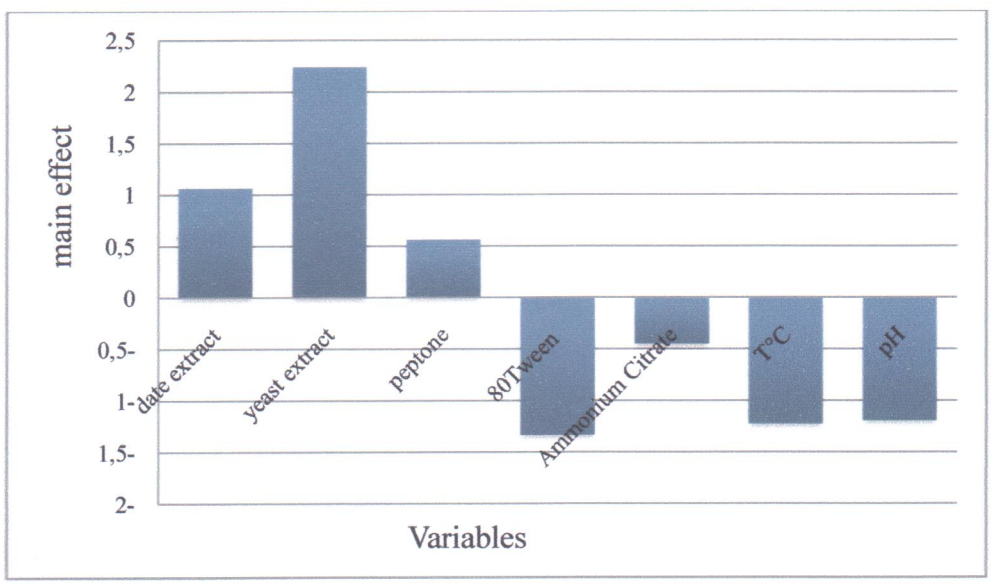

Figure 16: Effect of different factors on the biomass production.

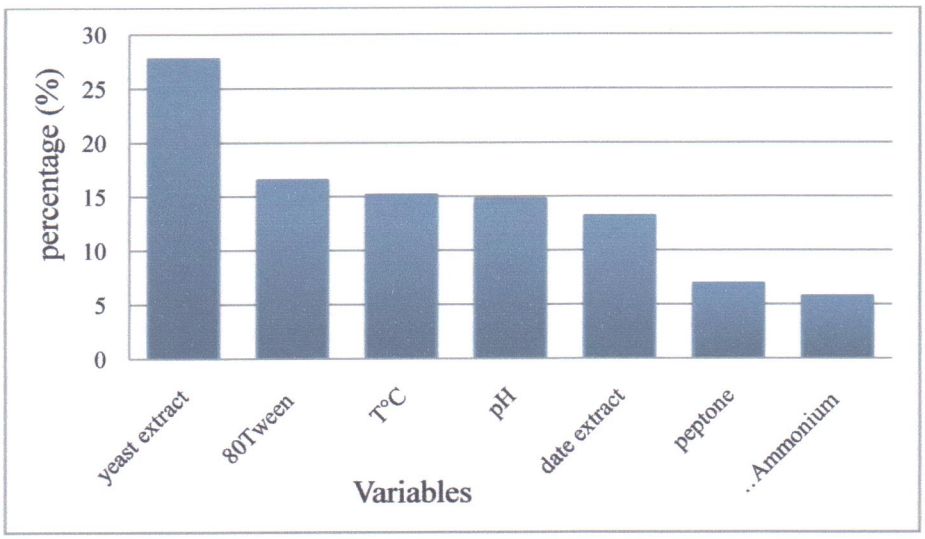

Figure 17: Pareto chart rationalizing the effect of each variable on the biomass production (g/l).

In other study, Sreekumar & Krishnan, (2010) used Plackett-Burman Design to enhance probiotic biomass production (*Bacillus subtilis* SK09), they found that pH, ammonium citrate and peptone were the most important variables. These key variables were selected for further optimization using RSM.

The calculated regression equation for the optimization of media components assessed the titer of biomass (Y, g/l) as a function of these variables. The following equation was found to explain biomass production:

$$Y_{biomass\ production} = 21,652 + 1,065X_1 + 2,24X_2 + 0,56X_3 - 1,335X_4 + 0,45X_5 - 1,225X_6 - 1,195X_7$$

IV.4.5.2.-Effect of medium components on antimicrobial activity

The Plackett Burman Design was applied in order to identify the significant factors in eight modified MRS medium that affect antimicrobial production. The Table (15) presents the Plackett-Burman design for seven variables and their corresponding response in terms of antimicrobial activity. Table 16 shows the regression analysis of the effect of each variable along with the coefficient level (%), t and P value.

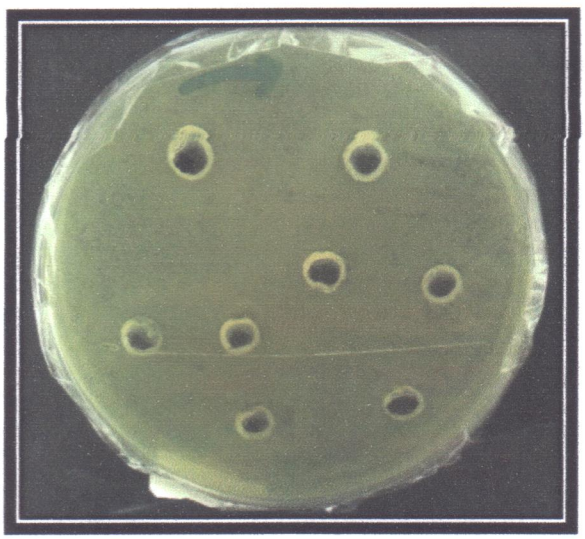

Figure 18: Results of different trials of Plackett-Burman design on antimicrobial activity.

Table 15: Plackett-Burman design for antimicrobial activity of *Lb. plantarum* G1

Trials	X1 (Date juice)	X2 (yeast extract)	X3 (Peptone)	X4 (Tween)	X5 (Ammonium citrate)	X6 (T°C)	X7 (pH)	Antimicrobial Activity(cm)
1	10(-1)	10(+1)	20(+1)	1(-1)	2(+1)	37(+1)	5.5(-1)	1.5
2	10(-1)	5(-1)	20(+1)	6(+1)	0.5(-1)	37(+1)	6.5(+1)	2
3	14(+1)	5(-1)	10(-1)	6(+1)	2(+1)	30(-1)	6.5(+1)	1.3
4	14(+1)	10(+1)	10(-1)	1(-1)	2(+1)	37(+1)	5.5(-1)	0
5	10(-1)	10(+1)	20(+1)	1(-1)	0.5(-1)	37(+1)	6.5(+1)	1.6
6	14(+1)	5(-1)	20(+1)	6(+1)	0.5(-1)	30(-1)	6.5(+1)	1.8
7	14(+1)	10(+1)	10(-1)	6(+1)	2(+1)	30(-1)	5.5(-1)	2.8
8	10(-1)	5(-1)	10(-1)	1(-1)	0.5(-1)	30(-1)	5.5(-1)	2.6

The data in Table (15) shows variation from 1.3 to 2.8 cm of antimicrobial activity. This variation reflects the importance of screening design to improve the production. The analysis of the data from the Plackett–Burman experiments involved a first order (main effects) model. The main effects of the examined factors on the antimicrobial activity were calculated and presented graphically in Figure (18).

Table 16: The regression analysis of the effect of each variable along with the coefficient level, t and P value

Variables	coefficient	*t-value*	*P-value*
Date juice (%)	-24,17	-16,6170094	0,03826518
Yeast extract (g/l)	0,77	10,2530483	0,94200725
Peptone (g/l)	14.19	0,07584614	0,06189502
Tween- 80 (g/l)	21.08	14,495689	0,0438484
Ammonuim citrate	-8.73	-6,01040764	0,10495814
T°C	-22.1	-15,2027958	0,04181494
pH	-8,14	-5,65685425	0,11138875

As it is clearly shown that the Tween-80 and yeast extract were found to be the most significant variables affecting antimicrobial activity they may be chosen for further optimization. The other variables of negative significant effect were not included in the next optimization experiment, but instead were used in all trials at their (-1) level (Sifour *et al.*, 2010).

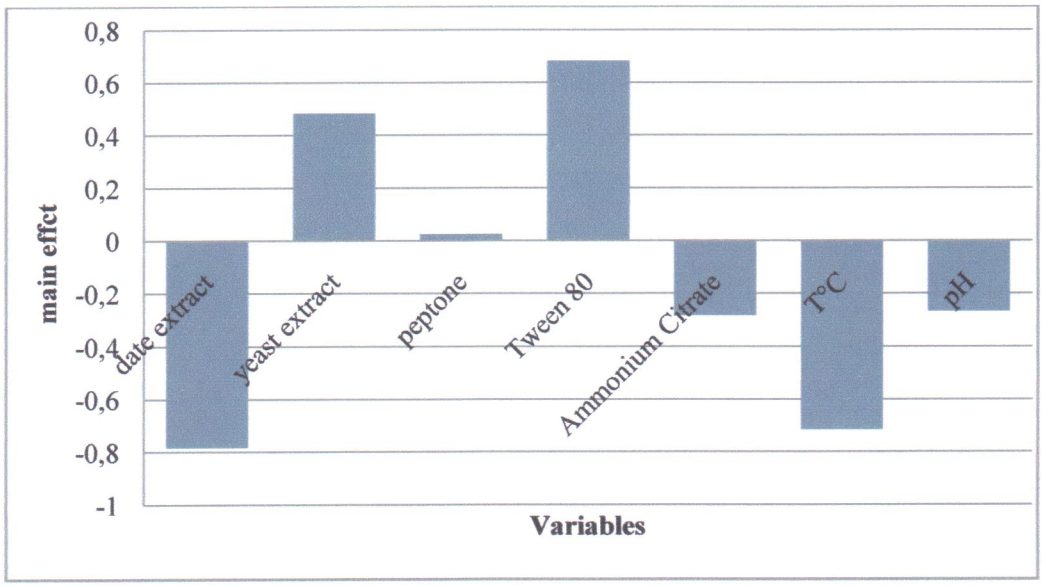

Figure 19: Effect of different factors on the antimicrobial activity

From the obtained results, it is clear that the variables that affect biomass production are not the same variables that effect antimicrobial activity. Despite of that the date juice is an ideal medium for *Lb. pantarum* G1 growth, it exerted a negative effect on antimicrobial activity. Figure (19) shows the ranking of factor estimates in a Pareto chart. The Pareto chart displays the magnitude of each factor estimate and it is a convenient way to view the results of a Plackett–Burman design.

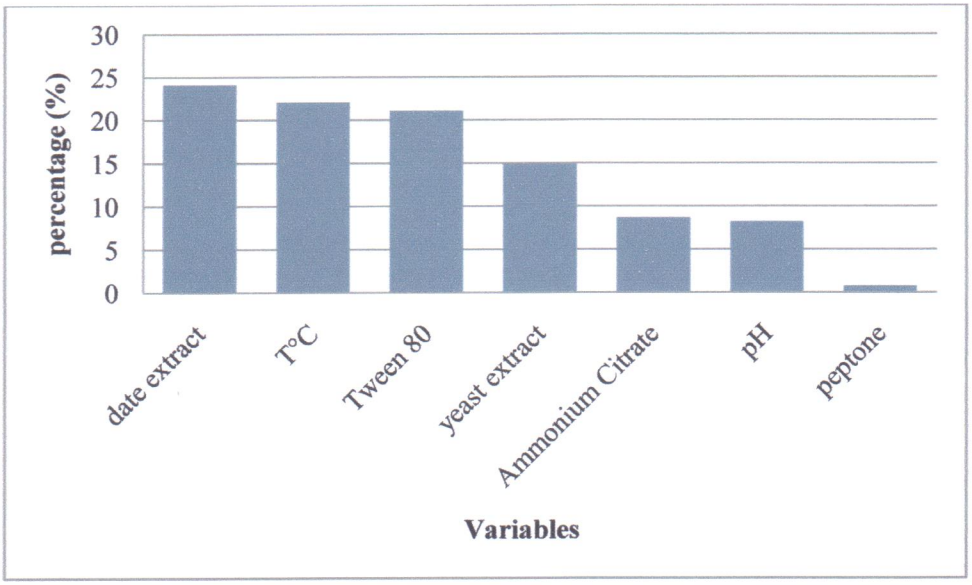

Figure 20: Pareto chart rationalizing the effect of each variable on the antimicrobial activity.

The polynomial model describing the correlation between the 7 factors and the biomass production could be presented as follows:

$$Y_{Antimicrobial\ activity} = 1,7 + 0,783X_1 + 0,483X_2 + 0,025X_3 - 0,683X_4 + 0,283X_5 - 0,716X_6 - 0,266X_7$$

Kaur *et al.*, (2013) applied Plackatt-Burman Design to screen the factors that effect bacteriocin production by *Pediococcus acidilactici*. They found that the KH_2PO_4 and peptone were the significant factors which have the positive effect.

IV.4.5.3.Effect of nutrient components on acidity

The same variables were screened for their effect on lactic acid production using the Plackett–Burman design. The design plan and the lactic acid production for the different trials are given in g/l and shown in Table (17).

Table 17: Plackett-Burman design of variables with acidity titer as response.

Trials	X1 (Date juice)	X2 (yeast extract)	X3 (Peptone)	X4 (Tween)	X5 (Ammonium citrate)	X6 (T°C)	X7 (pH)	Acidity g/l (%)
1	10(-1)	10(+1)	20(+1)	1(-1)	2(+1)	37(+1)	5.5(-1)	14.26
2	10(-1)	5(-1)	20(+1)	6(+1)	0.5(-1)	37(+1)	6.5(+1)	9
3	14(+1)	5(-1)	10(-1)	6(+1)	2(+1)	30(-1)	6.5(+1)	10.5
4	14(+1)	10(+1)	10(-1)	1(-1)	2(+1)	37(+1)	5.5(-1)	11.26
5	10(-1)	10(+1)	20(+1)	1(-1)	0.5(-1)	37(+1)	6.5(+1)	9.75
6	14(+1)	5(-1)	20(+1)	6(+1)	0.5(-1)	30(-1)	6.5(+1)	12.02
7	14(+1)	10(+1)	10(-1)	6(+1)	2(+1)	30(-1)	5.5(-1)	12.76
8	10(-1)	5(-1)	10(-1)	1(-1)	0.5(-1)	30(-1)	5.5(-1)	9

The variation in lactic acid production in different sets ranged from 9 to 14.26g/l reiterating the importance of selection and identification of important factors, the Pareto graph was drawn to show the effect of all variables on lactic acid production (Figure 21). The analysis of the data from the Plackett–Burman experiments involved a first order (main effects) model. The main effects of the examined factors on the lactic acid production were calculated and presented graphically in Figure (20).

Table 18: The regression analysis of the effect of each variable along with the coefficient level, t and P value

Variables	coefficient	*t-value*	*P-value*
Date juice (%)	8.74	3,56381818	0,17415566
Yeast extract (g/l)	29.56	12,0490996	0,05271465
Peptone (g/l)	13.04	1,55589241	0,17073571
Tween- 80	-3.46	-1,41421356	0,39182655
Ammonuim citrate (g/l	19.08	7,77817459	0,08140041
T°C	-12.14	-4,94974747	0,12690837
pH	-13.94	-5,68513852	0,11084574

From the results analysis of the regression coefficients of the seven variables peptone, ammonium citrate, yeast extract and date extract showed a positive effect on lactic acid production. These variables had confidence level above 95% in comparison to other variables and therefore, were considered to be highly significant for biomass production by *Lb. plantarum* G1. Temperature, pH and Tween-80 have a negative effect on lactic acid production from *Lb. plantarum* G1and Tween-80 have a slight effect on lactic acid productivity

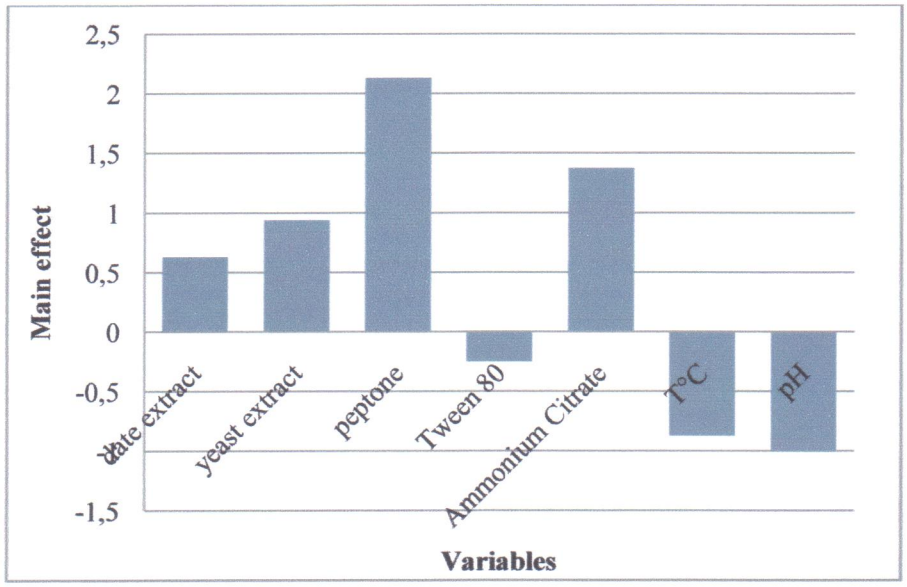

Figure 21: Effect of different factors on acidity (g/l)

On the basis of calculated t-values and confidence level (%) peptone was found to be the most significant variables affecting lactic acid production most significant followed by ammonium citrate, yeast extrait and date extract.

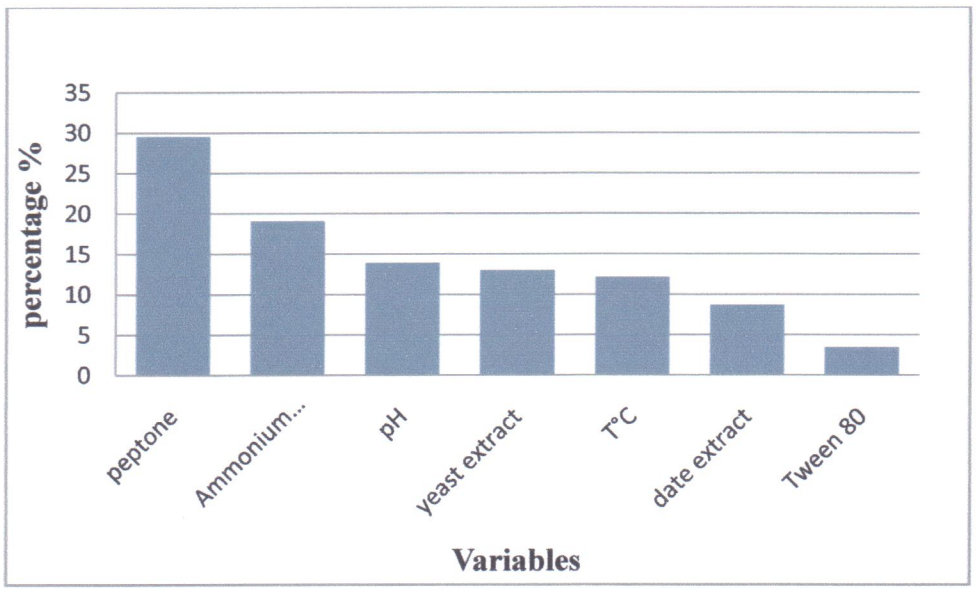

Figure 22: Pareto chart rationalizing the effect of each variable on acidity

The polynomial model describing the correlation between the 7 factors and that could be presented as follows:

$$Y_{Acidity} = 11{,}067 + 0{,}63X_1 + 2.13X_2 + 0.94X_3 - 0.25X_4 + 1.375X_5 - 0{,}875X_6 - 1.005X_7$$

Chauhan *et al.*, (2007) used Plackett-Burman Design with 15 variables to optimize lactic acid production from *Lactobacillus* sp. KCP01 using date juice as a sugar source, they found that the peptone, beef extract, yeast extract, K_2HPO_4, sodium acetate, sodium sulfate, $FeSO_2.7H_2O$, $MnSO_4.7H_2O$ and date juice were the significant factors that affect the production.

IV.4.5.4. Pre-optimization experiment

Table 19: pre-optimization experiment for evaluation of significant variables on biomass production.

Pre-optimized medium	Optical density (nm)	Biomass (g/l)	Antimicrobial activity (cm)	Acidity %
Pre-optimized medium I	1.014	21.98	1.15	8.63
Pre-optimized medium II	1.100	23.90	1.2	7.50
Pre-optimized medium III	0.975	21.11	1.1	8.25
Pre-optimized medium IV	1.102	23.95	1.3	9

The results obtained in Table19 confirm that the date extract and yeast extract were the important significant factors that affect biomass production. Biomass concentration obtained in the pre-optimized medium I and II indicate that the peptone was the variable that had a slight positive effect on biomass production. For antimicrobial activity, date juice had a negative effect, for this reason the antimicrobial activity is in same level. The results also indicate that peptone, yeast extract, date juice and ammonium citrate have a positive effect on lactic acid production. These variables which have a positive effect were chosen for further optimization.

Conclusion

Our experimental study focused on the optimization of *Lb. plantarum* G1 biomass production using date juice as a carbone source. This juice was prepared from date wastes collected from city of Ghardaia.

The physicochemical analysis indicated that the date juice is favorable to the growth of the bacterial strain because it contains a high amount of sugars easily assimilated. On the other hand, date juice is poor in protein and salts. For this reason it supplemented with yeast extract, $MgSO_4$, K_2HPO_4, KH_2PO_4 and Tween-80, and other components of MRS standard medium. The results showed that the date juice at 12% of total sugar concentration of 35 g/l supplemented with salts and organic nitrogen of MRS medium produce a higher biomass when compared with biomass production in MRS medium and in date juice alone.

Seven variables including date extract were screened for their effect on *Lb. plantarum* biomass production using Plackett-Burman design. Results from this analysis demonstrated that yeast extract, date extract and peptone were the most significant factor affecting biomass production.

The same model of Plackett-Burman was applied to estimate the effect of different factors on the production of antimicrobial agents from *Lb. plantarum* G1.The results showed that the Tween-80 and yeast extract were the most important factors that affect antimicrobial activity. For acidity, date juice, yeast extract, peptone and ammonium citrate was the significant variables.

Finally, we suggest that further studies could be carried out on optimization of biomass production by other approaches of optimization such as RSM (Response Surface Methodology) and we think that the use of date juice for probiotic biomass production during the fermentation and scale up in laboratory may give further ideas on potential of its use for industrial production.

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ANNEX I

Table 1: Preparation of the calibration curve of Glucose.

Dilutions	S	S/2	S/4	S/8	S/16	S/32	S/64	S/128
Concentrations (mg/ml)	5	2,5	1,25	0,625	0,3125	0,15625	0,078125	0,039062
DO (nm)	1,47	0,894	0,357	0,276	0,193	0,159	0,135	0,103

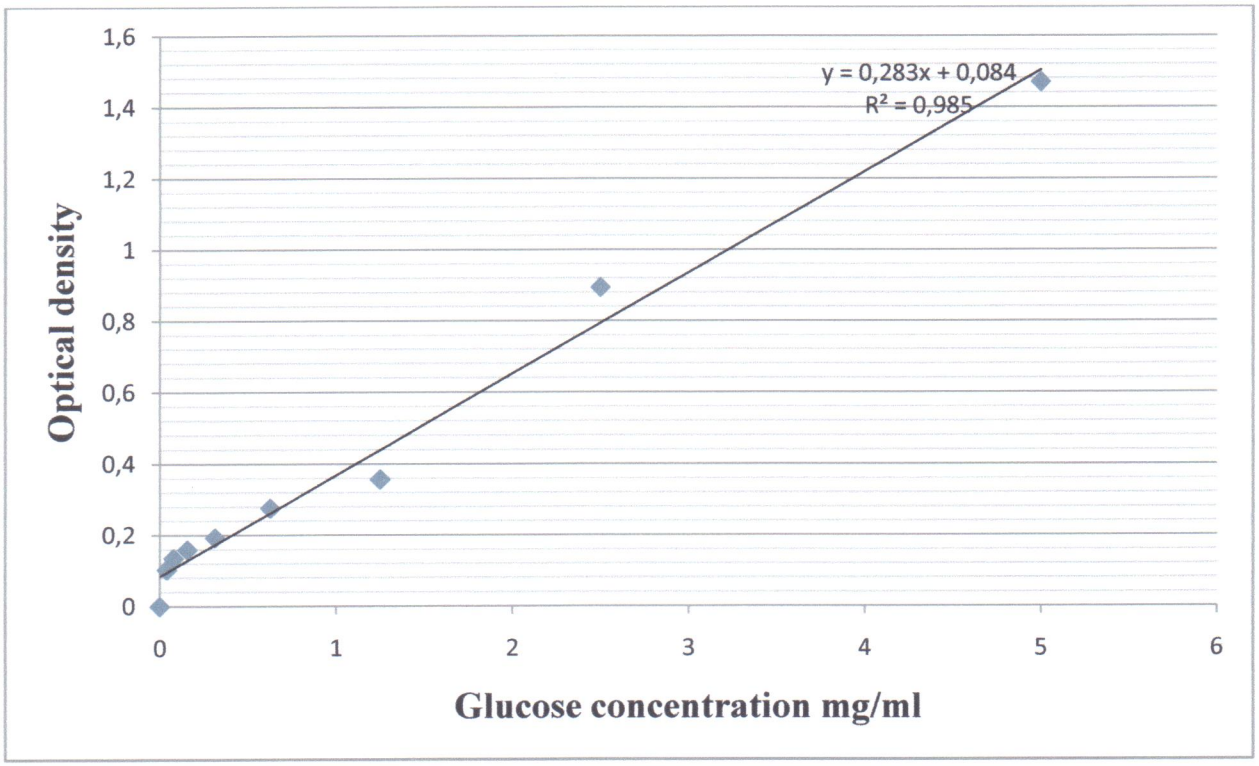

Figure 1: Calibration curve of Glucose

ANNEX II

Table 2: Preparation of the calibration curve of gallic acid

Dilutions	S	S/2	S/4	S/8	S/16	S/32
Concentrations (mg/ml)	0,0625	0,03125	0,015625	0,0078125	0,00390625	0,00195313
DO (nm)	0,925	0,463	0,198	0,075	0,049	0,018

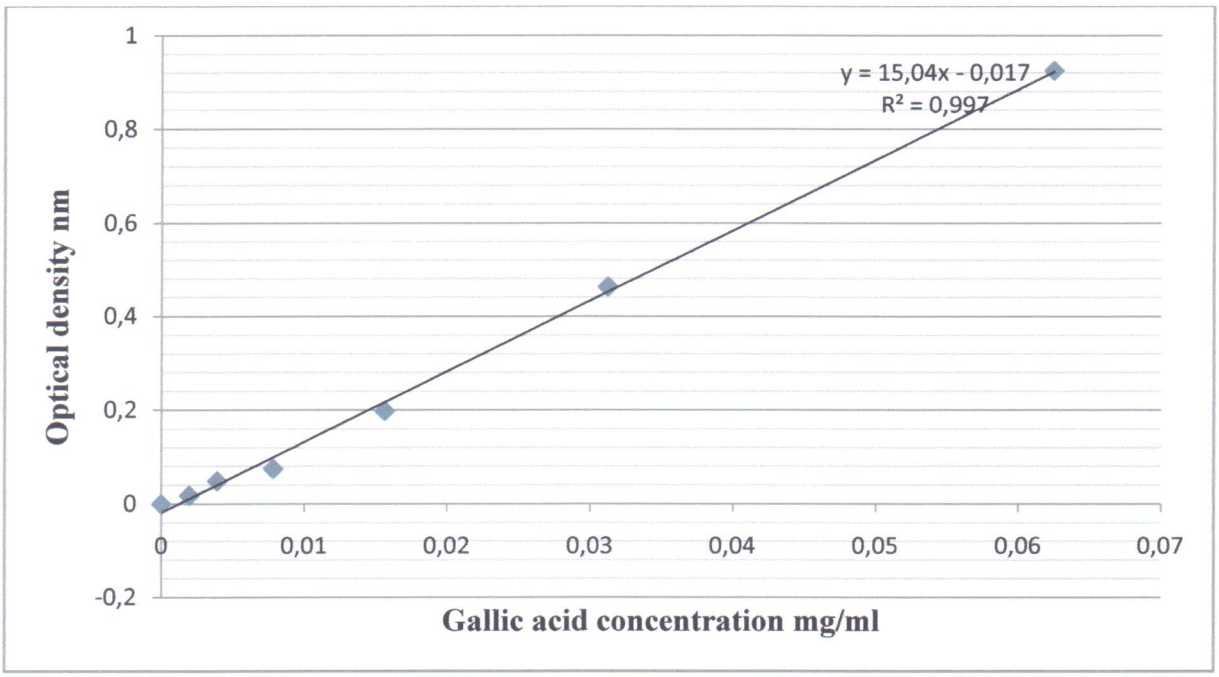

Figure 2: Calibration curve of gallic acid.

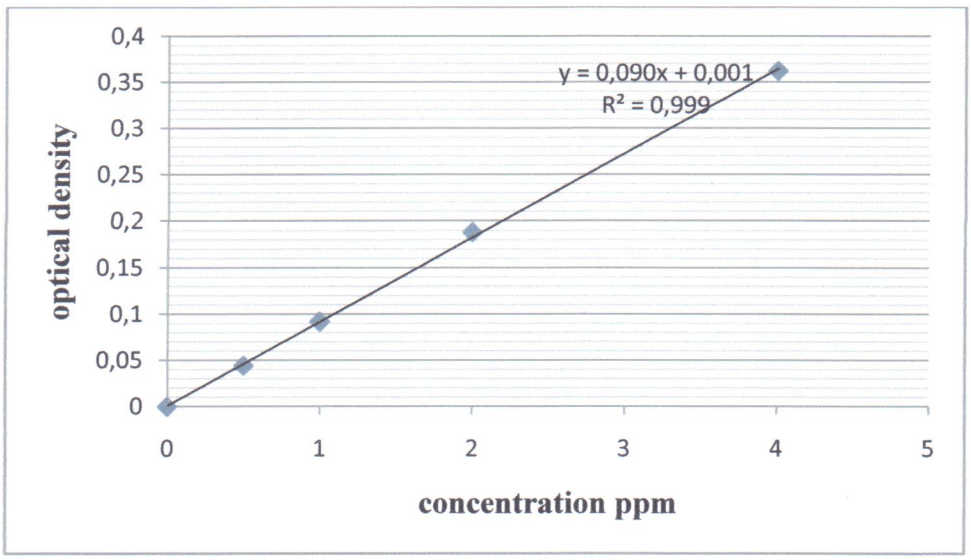

Figure 3: Calibration curve of Manganese

ANNEX III

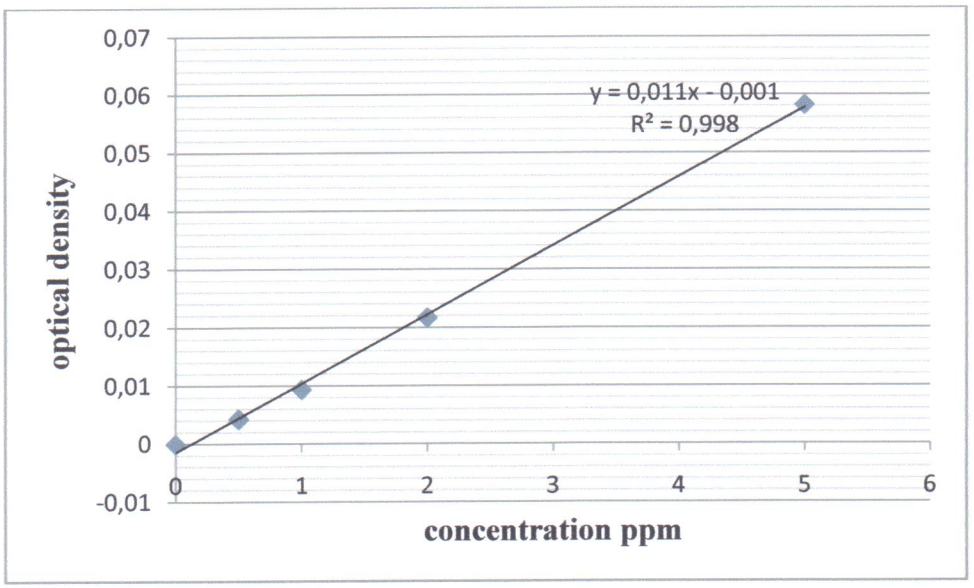

Figure 4: Calibration curve of Lead

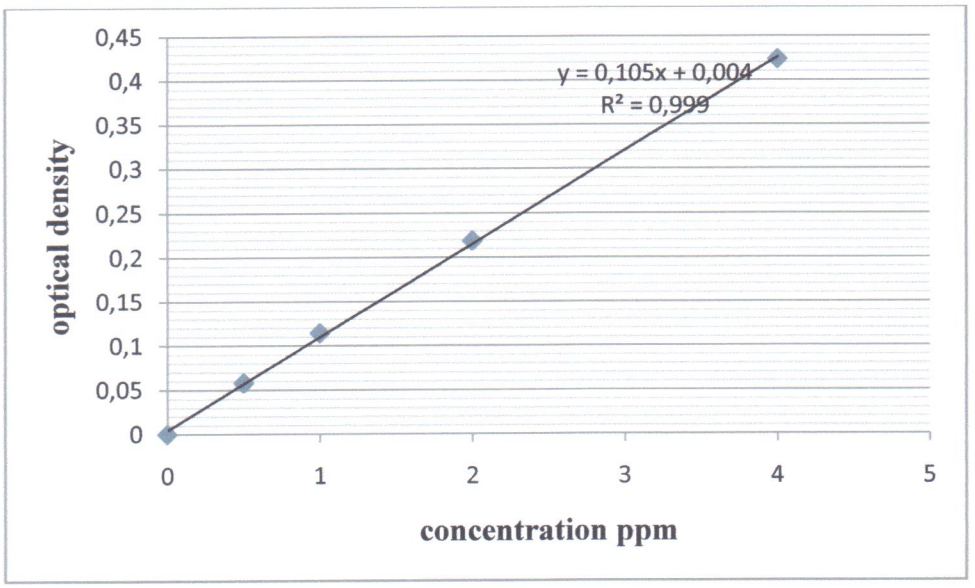

Figure 5: Calibration curve of Copper

ANNEX VI

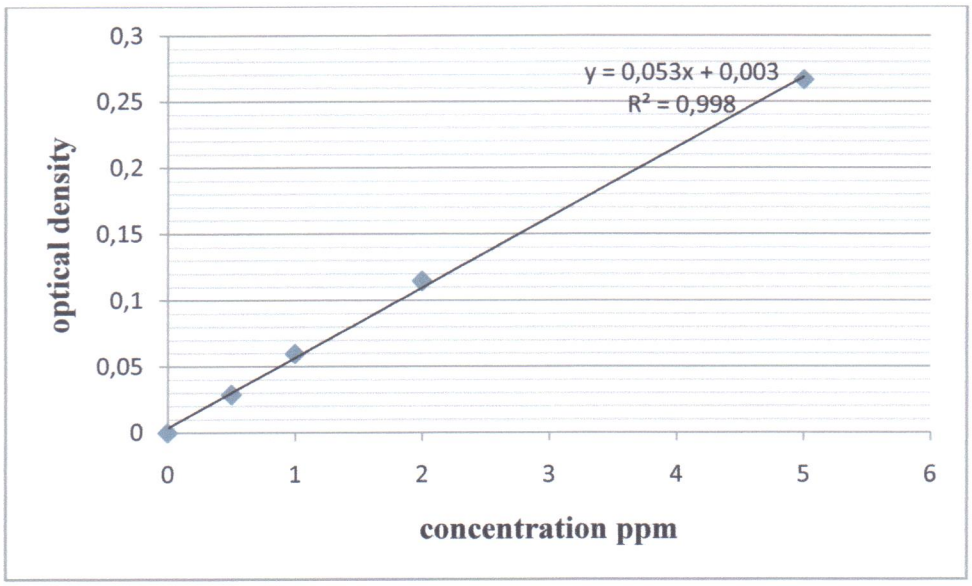

Figure 6: Calibration curve of Iron

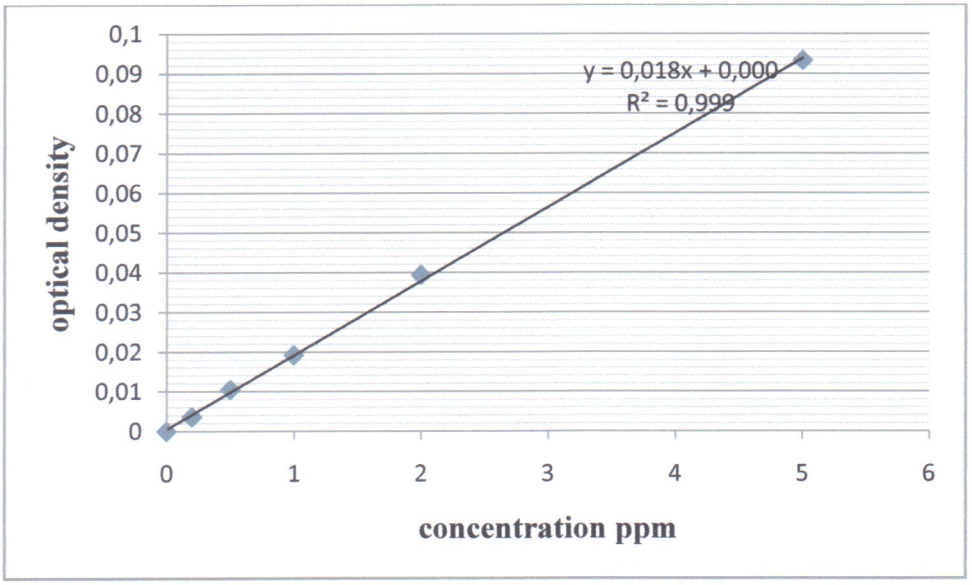

Figure7: Calibration curve Chrome

ANNEX V

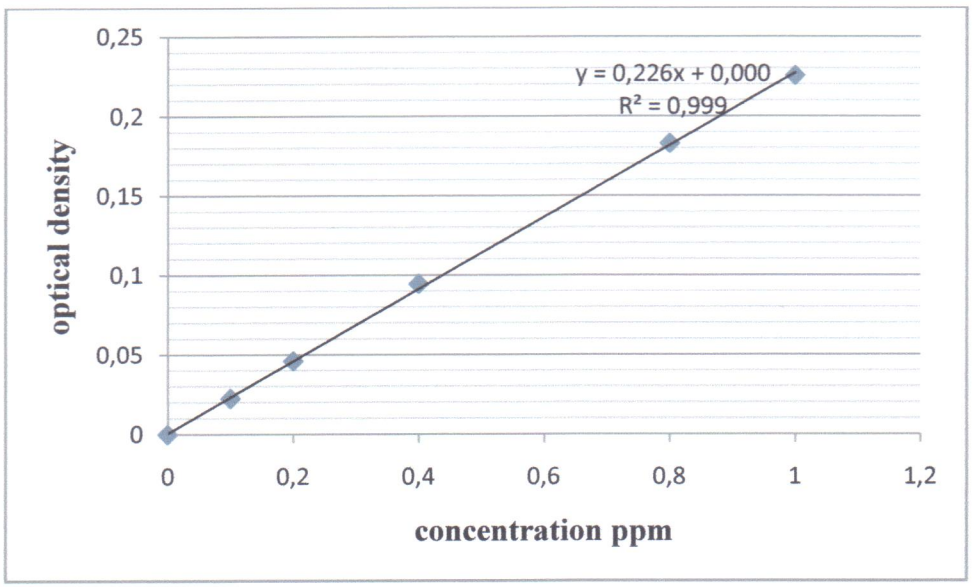

Figure8: Calibration curve of Zinc

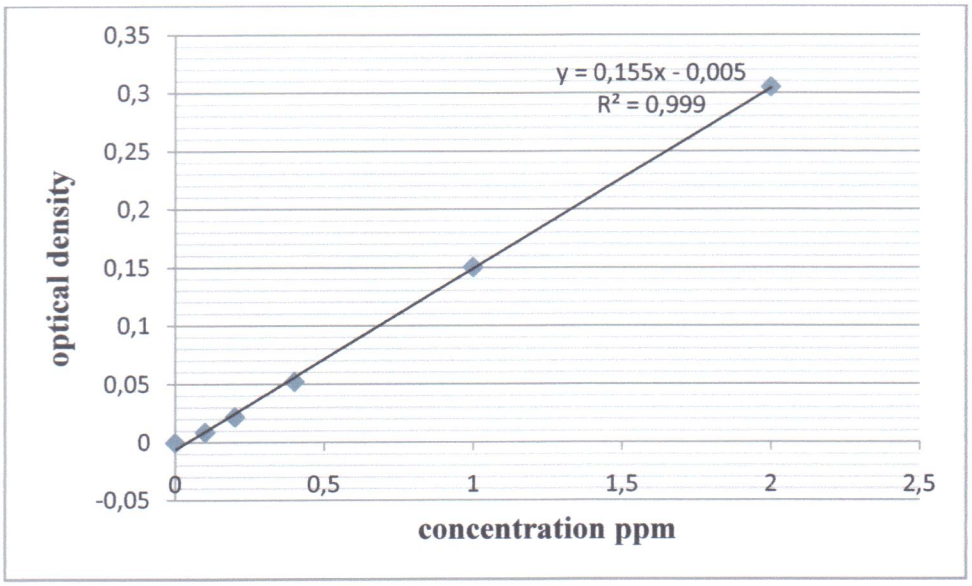

Figure 9: Calibration curve of Cadmium

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Examiner: Miss. Amira Samiya

Supervisor : Dr. Sifour Mohamed

Theme

Optimizing the production of probiotic biomass in the presence of date extract

Abstract

The increasing demand for products containing probiotic microorganisms from health-conscious consumers need to establish effective fermentation techniques for probiotic biomass production. Production of probiotics should be based on the cost of nutrient substrates and other parameters. To reduce this cost, it is desirable to use low-cost raw materials.

This study was conducted to valorize date waste in order to improve biomass production of *Lactobacillus plantarum* G1 using date extract as carbon source. The culture medium was optimized using statistical experimental design of Plackett-Burman. A two-level Plackett-Burman design was adopted to screen the most important nutrients influencing biomass production, which showed that the date extract, yeast extract and peptone were the most significant factors.

Keywords: **probiotic, *Lactobacillus plantarum* G1, biomass production, date extract, Plackett-Burman design.**

Résumé

La demande croissante pour les produits contenant des microorganismes probiotiques auprès des consommateurs soucieux de leur santé doivent mettre en place des techniques de fermentation efficaces pour la production de biomasse probiotique. La production des probiotiques devrait être basée sur le coût des substrats nutritifs et d'autres paramètres. Pour réduire ce coût, il est souhaitable d'utiliser des matières premières moins chères.

Cette étude a été menée pour valoriser les déchets de dattes afin d'améliorer la production de biomasse de *Lactobacillus plantarum* G1 en utilisant l'extrait de datte comme source de carbone. Le milieu de culture a été optimisé en utilisant des modèles statistiques expérimentaux de Plackett-Burman. Une matrice de Plackett-Burman à deux niveaux a été adoptée pour sélectionner les nutriments les plus importants qui affectent la production de biomasse, qui a montré que l'extrait de datte, l'extrait de levure et peptone sont les facteurs les plus significatifs.

Mots clés: **probiotique, *Lactobacillus plantarum* G1, production de biomasse, extrait de datte, Plackett-Burman design.**

الملخص

ان تزايد الطلب على المنتجات التي تحتوي على الكائنات الحية الدقيقة البروبيوتكية من طرف المستهلكين الواعين بصحتهم بحاجة إلى إنشاء تقنيات تخمير فعالة لإنتاج الكتلة الحيوية البروبيوتكية وينبغي أن يستند في إنتاجها على تكلفة المغذيات وغيرها من المعايير.لخفض هذه التكلفة من المستحسن استخدام مواد خام ذات تكلفة منخفضة.

أجريت هذه الدراسة من أجل استغلال مخلفات التمر لتحسين إنتاج الكتلة الحيوية لبكتيريا ***Lactobacillus plantarum* G1** باستخدام مستخلص التمر كمصدر للكربون. تم تحسين وسط الزرع باستخدام تصاميم إحصائية تجريبية. اعتمد على مصفوفة **Plackett-Burman** ذات مستويين لتعيين العناصر الغذائية التي تؤثر على إنتاج الكتلة الحيوية، والتي أظهرت أن مستخلص التمر ، مستخلص الخميرة والبيبتون هي العوامل الأكثر أهمية.

الكلمات المفتاحية : البكتيريا البروبيوتكية،إنتاج الكتلة الحيوية ، مستخلص التمر، مصفوفة Plackett-Burman