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Exploring the potential of soapstock over a glycerol in vitamin K2 production by *Bacillus subtilis* natto: a comparative analysis

Faranak Ansari[1], Hoda Nouri[2] and Hamid Moghimi[1*]

Abstract

Background Vitamin K2 is an essential nutrient for blood coagulation and cardiovascular health and mainly produced by bacteria strain like *B. subtilis*. researchers have explored producing strain improvement, cultivation mode, environmental optimization, increased secretion, and using cheaper carbon and nitrogen sources in order to increase vitamin K2 productivity. This study examines the impact of varioius concentration of soapstock, which is a by-product of vegetable oil refining, as an alternative carbon source with lower pirce, in the fermentation medium instead of glycerol on the microbial synthesis of vitamin K2 using *B. subtilis* natto ATCC 23857.

Results The results demonstrate that when the glycerol in fermentation medium was substituted with soapstock, by 75% concentartion, the fermentation process produced a yield of 158.16 mg/L of vitamin K2 after 72 h; This was 3.8 times more than the control medium containing glycerol. When the entire culture medium was replaced with wastewater, the vitamin K2 concentration reached 21.18 mg/L, 52% of the control medium's concentration. If the carbon sources in the fermentation medium consisted of 20% soapstock and 47.4 g/L glycerol (maintaining the same final glycerol concentration as the control medium), the vitamin K2 concentration reached 35.7 mg/L or 85.8% of the control medium. The analysis of soapstock fermentation medium characteristics reveals that after fermentation with *B. subtilis*, the COD of soapstock fermentation medium was dramatically reduced from 259,500 mg/L to 57,830 mg/L.

Conclusions Using soapstock as an alternative carbon source for fermentation did not negatively impact the bioprocess and increased vitamin K2 production. Therefore, this research introduces an alternative carbon resource for vitamin K2 production and paves the way for the biorefinement of soapstock.

Keywords *Bacillus subtilis* natto, Carbon source, Crude glycerol, Soapsotck, Vitamin K2

Introduction

Vitamin K2 is a member of the fat-soluble Vitamin K group, which is essential for blood coagulation, osteoporosis prevention, and cardiovascular health [1–3]. Also, known as menaquinone (MK) [4], and comes in several forms, ranging from MK-2 to MK-14 [5], among all these, MK-7 is particularly noteworthy for its high bioavailability and extended half-life making it more effective than other vitamin K2 homologs [6, 7]. Various microorganisms produce menaquinone, an important electron carrier in the respiratory chain of microbial cells [8, 9]. Bacteria are the predominant producers [10]

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of menaquinone, and different strains can produce it in various forms. The production of vitamin K2 in the MK-7 form by *B. subtilis* can reach up to 96% of the total production [11, 12]. *B. subtilis* is a suitable candidate strain for the microbial production of vitamin K2 owing to its consideration as (Generally Recognized As Safe) organism. Also, *B. subtilis* is notable for its high production of MK-7, compared to other menaquinone producers [13, 14]. Many experiments have been conducted to increase vitamin K2 productivity and accelerate its industrial production [14–16]. These include strain improvement [17, 18], different environmental optimization such as nitrogen sources, carbon sources, temperature [15], pH [19], biofilm formataion [20], dissolved oxygen [21], exploring different cultivation strategies and innovative bioreactor such as biofilm reactor [22], increased secretion [23], and using more affordable options for carbon and nitrogen sources [24, 25]. Carbon and nitrogen sources are influential factors in vitamin K2 production. Result from previous study on determining potential carbon sources in mk-7 production reveals that among glycerol, glucose, sucrose and starch, glycerol is the most significant factor on MK-7 synthesis among all factors studied [15]. MK-7 production often has low yields and expensive production costs; some products could take the place of pricey raw ingredients in it [26]. In order to reduce production costs, using a cheaper carbon source such as crude glycerol is considerable [27]. Crude glycerol is a by-product of biodiesel production through transesterification, soap manufacturing via saponification, and hydrolysis reactions [28]. Another source of crude glycerol is soapstock. Soapstock is a by-product of refining crude or degummed vegetable oils. During this process, oils are treated with alkali to produce sodium soap. Enzyme production, biodegradation, and biodiesel production are some of the major uses of soapstock as a valuable biotechnological resource [29, 30]. The use of alternative, economical carbon sources has attracted the attention of researchers in recent study, to utilize these alternative carbon sources for MK-7 production, a mutant strain of *Bacillus amyloliquefaciens* capable of digesting maize meal was developed, resulting in a high MK-7 content [31]. Wang et al. used liquefied soybean flour and corn flour as primary carbon sources, achieving an MK-7 production yield of 18.9 mg/mL in shaking flask culture [32]. This study used soapstock as an alternative carbon source instead of glycerol for the first time to develop an economical fermentation medium for vitamin K2 in MK-7 form. This approach not only reduces production costs but also advances the biorefinery process and promotes a circular economy.

Material and methods

Bacterial strain and culture conditions

B. subtilis natto ATCC 23857 was used in this study as a producing microorganism and was stored on nutrient agar plates and fresh plates cultivated monthly. One loop of bacteria from the fresh nutrient agar plate was inoculated to pre-culture media containing beef extract 5 g/L, glucose 30 g/L, peptone 40 g/L, NaCl 5 g/L, and yeast extract 5 g/L. For soapstock's pre-culture medium, soapstock was diluted with distilled water in a 1:9 (v/v) ratio. The fermentation process was carried out under shaking conditions at 120 rpm and 37 °C for 24 h in a shaking incubator.

The control fermentation medium for vitamin K2 production was based on a basal medium. This medium consists of 50 g/L of glycerol, 30 g/L of soybean peptone, 0.6 g/L of yeast extract, 0.3 g/L of K_2HPO_4, 0.3 g/L of $MgSO_4.7H_2O$, and 0.1 g/L of $CaCl.H_2O$, used as a control medium according to Zhang's study [27]. The soapstock fermentation media contained 1.3 to 13 g/L of soapstock, 30 g/L of soybean peptone, 0.6 g/L of yeast extract, 0.3 g/L of K_2HPO_4, 0.3 g/L of $MgSO_4.7H_2O$, and 0.1 g/L of $CaCl.H_2O$. The control and soapstock fermentation media were seeded at a rate of 5% with a 24-h pre-culture of soapstock or control media. These flasks were then placed in a rotary shaker under the conditions of 210 rpm and 37°C because vitamin K2 production was boosted under higher agitation rate conditions [21], and this continued until the death phase was detected. Samples were taken every 12 h to measure biomass, pH, absorbed glycerol, and vitamin K2 yield. All media's pH were adjusted at 7 before cultivation.

Soapstock characteristics

Soapstock was provided by Varamin oil-extracting factory No.1 Iran. The glycerol content of soapstock was measured at 13 g/L measured by spectrophotometric methods, pH 10.38 was measured by pH meter, COD 25050 mg/L was measured with the COD kits, TDS 2400 mg/L was measured by the TDS meter, and fatty acid measured to be 16% (w/v) by liquid–liquid extraction.

Soapstock preparation

In the first attempt to produce vitamin K2 using soapstock, glycerol was partially replaced in the control medium by adding soapstock at the following concentrations: 10%, 20%, and 30%. In all batches the substitution of glycerol with an equivalent of its concentration in soapstock, allowed the final glycerol content to remain at 50 g/L. More details are provided in Table 1 based on the varying results from different glycerol concentrations, this research conducted to find out how diffrent

Table 1 Glycerol and soapstock mixing ratio

Name	Glycerol provided from soap stock concentration (g/L)	Control concentration (g/L)	Total glycerol concentration (g/L)
Batch 10% + Gly	1.3	48.7	50
Batch 20% + Gly	2.6	47.4	50
Batch 30% + Gly	3.9	46.1	50

soapstock concentrations affected the formation of vitamin K2. Therefore, in the next step, glycerol was eliminated, and soapstock was used as the sole carbon source, diluted with distilled water in the following ratios: 10:90, 20:80, 30:70, 50:50, 75:25, and ultimately, 100% soapstock was used in the fermentation medium.

Measurement of bacterial growth and residual glycerol concentration

The spectrophotometric approach was used to detect free glycerol based on the oxidation of glycerol and the production of formaldehyde after it reacted with acetylacetone, resulting in a yellow compound measured at 410 nm [33] using a Shimadzu UV-1601 device. The dry cell weight was determined by precipitating cells from a 10 mL fermentation broth sample and drying them overnight at 105 °C [34].

Determination of MK-7

After bacterial growth, 10 mL fermentation broth was provided to sterile centrifuge tubes, then biomass and supernatant were separated using a centrifuge set at 6000 Rpm for 10 min. After that, supernatant was transferred to a sanitized flask in preparation for the vitamin K2 extraction process.

For extracting the vitamin K2 from the aqua phase of the medium the mixture of n-hexane and isopropanol was added to the supernatant (4:2:3, v/v) and the organic phase shaped. Phases were separated by centrifuging the extraction mixture for 10 min at 6000 Rpm after it had been incubated for an hour at 30°C in a shaker incubator. In the next step 5mL of solvent layer extracted from the flask and transferd to a rotatory evaporator for 10 min at 40 °C. Then evaporator flask washed with 1mL of HPLC-grade ethanol to obtain MK-7 extract [35].

HPLC analysis

Vitamin K2 was quantified using an Agilent 1260 infinity high-performance liquid chromatography system. The following were the HPLC's operating conditions for the chromatographic column: SB-C18 4.6*250, SB-C18 guard cartridge 4.6*12.5/pk; mobile phase: 97% methanol: 3% water; flow rate: 1.0 mL/min; column temperature: 25°C; and detection wavelength: 268 nm [36]. The MK-7 calibration curve was linear between 5 mg/L and 100 mg/L ($R^2 = 0.997$).

Soapstock parameters determination

Soapstock parameters, including chloride, nitrite, phosphate, ammonium, sulfite, and total hardness, were measured before and after the fermentation with Vaheb analytical kits. Also, the Total Dissolved Solid (TDS) was measured by the TDS meter, and the Total Suspended Solids (TSS) was determined After filtering and drying at 105°C following the standard method 2540 B [37]. The chemical oxygen demand (COD) was measured with the COD kit, which is close to the standard method 5220 B-open reflux titrimetric [38], and using the Hanna HI83314 COD photometer for reading. The determination of total fatty acid was performed by the liquid–liquid extraction with n-hexane, as recommended by EPA Method 1664 [39].

Statistical analysis

The statistical software used for testing the result was SPSS Version 27.0.1, and the analysis was conducted in triplicate, also in this study, one-way ANOVA and Tukey's HSD tests were employed and set with a 95% confidence level, and the significance level was reported as $p \geq 0.05$.

Result and discussion

Determination of kinetics of biomass, glycerol, and vitamin K2 concentration

To examine the effect of soapstock concentration on vitamin K2 production, the kinetics of biomass, glycerol consumption, and vitamin K2 concentration were studied in both a control fermentation medium and a soapstock fermentation medium (Fig. 1).

According to the kinetics shown in Fig. 1a, *B. subtilis,* was in its exponential growth phase from 12 h until 36 h in the control medium. while, in the soapstock medium, it took 48 h to observe this phase, and most of glycerol consumption occurred during this phase in the both medium. Biosynthesis of vitamin K2 began after 24 h of fermentation in the control medium. however, during the first 24 h of fermentation in the soapstock medium, no vitamin K2 was detected, and it required

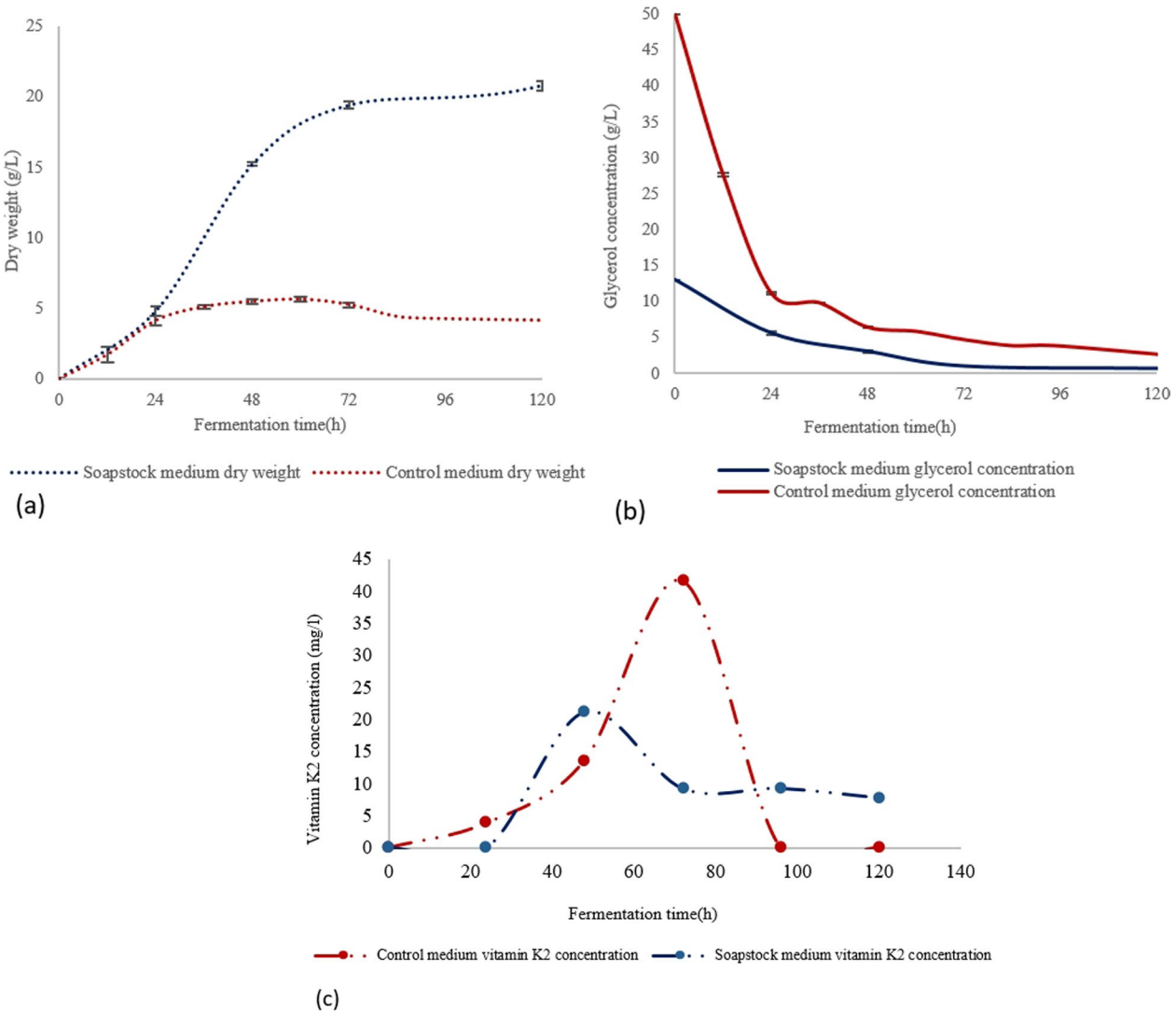

Fig. 1 Comparison of kinetics of biomass, glycerol consumption, and vitamin K2 production in control medium and soapstock fermentation medium. **a** Dry weight, **b** Residual glycerol concentration, and **c** Vitamin K2 concentration

48 h of fermentation to reach its maximum concentration. The biomass reached its highest concentration of 20.8 g/L at the 120 th hour with soapstock medium and compared to the control medium it was 3.7 fold higher. It indicates that *B. subtilis* was provided with a secondary carbon source from the soapstock medium, which is a fatty acid. This resulted in increased biomass growth even after most of the glycerol in the medium had been consumed. The ability of *B. subtilis* strain to degrade hydrocarbons [40] and bioremediate oily wastewater has been reported [41]. Additionally, the lipolytic activity of *B. subtilis* improved by using inexpensive vegetable oils [42]. Compared to stationiry phase, it has been reported, MK-7 production mainly observed in growth phase [15], while the highest concentration of vitamin K2 in the control medium reached at 41 mg/L in 72 h. This results were in agreement with litature report indicating that MK-7 production continues in spite of glycerol depletion [43]. However, Zhang's results showed no significant differences in bacterial growth or MK-7 synthesis between pure and crude glycerol-containing media, suggesting that crude glycerol can be replaced with pure glycerol as a lower-cost carbon source [27]. Whereas in the soapstock medium, the MK-7 concentration reached 21.8 mg/L in the 48 th hour of fermentation and decreased after the logarithmic phase. This drop in production might be due to the differences in the components of these two media, as glycerol's efficiency in transferring through the cell

membrane contributes to some advantages in the MK-7 production process [44].

Soapstock as a partial replacement for glycerol

Based on the result obtained from the comparison between the control medium containing glycerol and soapstock medium, both partial replacement of glycerol with soapstock and dilution of soapstock were examined. In the first step, a partial replacement for glycerol was such that, the total glycerol concentration in the fermentation medium matched that of the control medium, and it contained both glycerol and soapstock in a specific ratio that maintaining a total glycerol concentration of 50 g/L (Table 1); The kinetics of glycerol consumption and biomass production were assessed to determine the optimal batch for vitamin K2 analysis, as shown in Figs. 2 and 3.

In batch 10% + Gly, exponential growth was observed at 36 h, similar to the control fermentation medium, and glycerol consumption increased rapidly until the 48th hour. Batch 30% + Gly the death phase occurred in the 96th hour of fermentation, with the highest biomass of 7.07 g/L observed at 60 h (Fig. 2a,b). Batch 20% + Gly showed a more balanced glycerol consumption and the

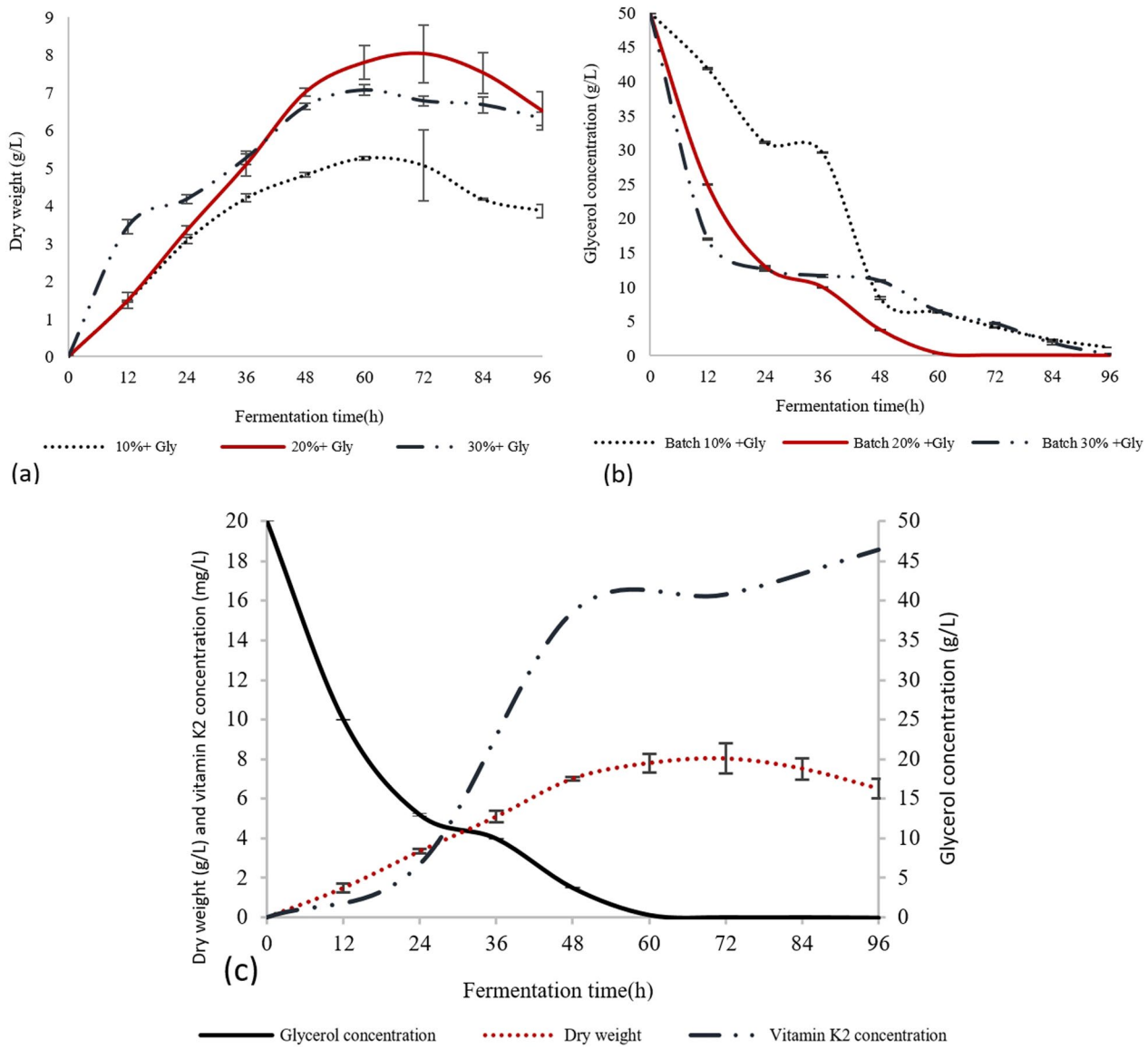

Fig. 2 Comparison of the kinetics of biomass, glycerol consumption, and vitamin K2 production in batches 10% + Gly, 20% + Gly, and 30% + Gly. **a** Dry weight. **b** Glycerol consumption. **c** Kinetics of biomass, glycerol concentration and vitamin K2 concentration in batch 20% + Gly

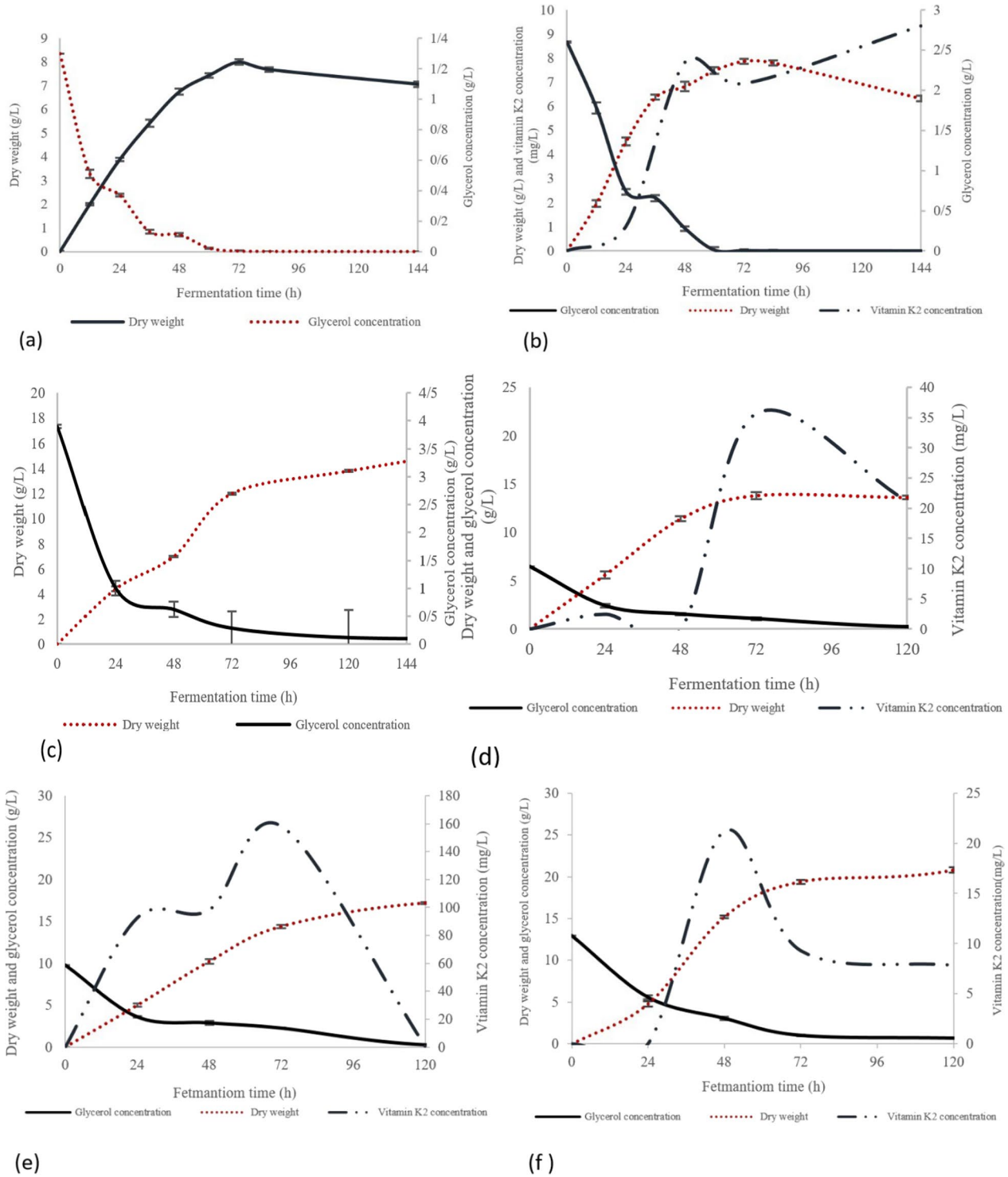

Fig. 3 Kinetics of biomass, glycerol consumption, and vitamin K2 production in different soapstock concentrations. **a** Kinetics of biomass, glycerol consumption with 10% (v/v) soapstock, **b** Kinetics of biomass, glycerol consumption and vitamin K2 concentration with (v/v) 20% soapstock, **c** Kinetics of biomass, glycerol consumption with (v/v) 30% soapstock, **d** Kinetics of biomass, glycerol consumption and vitamin K2 concentration with (v/v) 50% soapstock, **e** Kinetics of biomass, glycerol consumption and vitamin K2 concentration with (v/v) 75% soapstock, **f** Kinetics of biomass, glycerol consumption and vitamin K2 concentration with (v/v) 100% soapstock

highest biomass concentration, compared to the other batches. Consequently, it was the preferred choice for measuring vitamin K2 production due to the result from literature suggesting addition of excessive glycerol has no effect on MK-7 production, as microorganisms cannot utilize it [45]. According to reports, using glycerol and another carbon source at the same time can cause glycerol to act as an activator during the production of MK-7. [16]. In batch 20% + Gly, the maximum production of vitamin K2 was 18.57 mg/l in the 96th hours of fermentation (Fig. 2c). Glycerol may act as an enhancer in MK-7 production without impacting cell growth [46]; This result indicates that soapstock addition improved cell growth while not significantly affecting MK-7 production compared to the control medium.

Effect of different soapstock concentrations on vitamin K2 production

Considering that soapstock addition as a carbon source influenced both biomass and vitamin K2 production compared to the control medium, further experiments were conducted to assess the impact of varying soapstock concentrations without added glycerol. The kinetics of biomass, vitamin K2 content, and glycerol consumption are shown in Fig. 3.

In batch 20% soapstock, with 2.6 g/l glycerol, the highest production of vitamin K2 was measured at the 144th hour from the initiation of fermentation, with a concentration of 9.44 mg/l, also fermentation time was extended from 96 to 144 h due to an increase in soapstock concentration, which provided more access to carbon sources and delayed the death phase of *B. subtilis* natto (Fig. 3b). Batch 30% was not analyzed for vitamin K2 production due to its similarity to the fermentation process of the previous culture (Fig. 3c). In batch 50%, similar to the control fermentation medium, the highest production of vitamin K2 of 35.7 mg/L was achieved at 72 h, the measurement of vitamin K2 concentration continued until 120th hour, at the point which it decreased to 21.2 mg/L (Fig. 3d). Batch 75% soapstock, with 9.75 g/L of glycerol, achieved the highest vitamin K2 concentration of 158.16 mg/L. This is maximum vitamin K2 yeild among all batches (Fig. 3e). The addition of glycerol enhances MK-7 biosynthesis by optimizing viscosity and water activity, which improves mass transfer [47] consequently, the increase in MK-7 observed in this batch may be attributed to these effects. Batch 100%, with 13 g/L of glycerol, reached a peak vitamin K2 concentration of 18.21 mg/L (Fig. 3f). After 36 h of fermentation, *B. subtilis* natto exited the exponential growth phase and entered the stationary phase. Compared to the control, vitamin K2 production was enhanced in the soapstock fermentation medium, especially with a 75% soapstock concentration. In a similar study, crude glycerol was used as a carbon source to produce vitamin K2 by *B. subtilis* Z-15 economically and confirmed that the usage of crude glycerol didn't affect either the synthesis of vitamin K2 or the growth of *B. subtilis* and using crude glycerol instead of control resulted in a 70% cost saving, too [27]. The impact of using wheat starch wastewater instead of glycerol as an extra carbon source has been investigated. This substitution successfully reduced the cost of the carbon source by 33%, the price of the nitrogen source by 7%, and the cost of the phosphate source by 100% [48]. The vitamin K2 production yield in different alternative carbon sources is provided in Table 2. All these studies successfully took advantage of utilizing alternative carbon sources strategy as a cost-effective substrate for economical MK-7 production. This research has the highest concentration of vitamin K2 compared to other studies using alternative carbon sources, being 3.5 times higher than Zhang's research [27] and 2.5 times higher than Xu's [31]. The maximum value for vitamin K2 production was reported to be 226 mg/L, using glycerol as the carbon source [21], which is 1.4 times higher than the rate in this study.

Vitamin K2 purification is complex and costly, and it becomes even more complex when soapstock is employed in its production bcause of intensifying the purification challenges. There are potential solutions to this issue, including the utilization of complete fermented medium as a product. This strategy successfully implemented by Novin et al. by introducing a fermented dairy product containing vitamin K2, and effectively eliminating the downstream process for vitamin K2 purification [31]. This fermented product can be used as an animal supplement with some pre-treatment for soapstock.

Table 2 Yield of vitamin K2 in fermentation medium containing alternative carbon sources

Author/year	Carbon source	Yield	Strain	Refs.
Zhang/2020	Crude glycerol (a byproduct of biodiesel)	45.11 ± 0.62 mg/L	*B. subtilis* Z-15 (CICC 10260)	[27]
Zhang/2020	Liquefied wheat flour	41.43 mg/L	*B.subtilis* W-17	[44]
XU/2017	Maize meal	61.3 ± 5.2 mg/L	*B. amyloliquefaciens* H.β.D.R.-5	[31]
This research	Soapstock	158.16 mg/L	*B. subtilis* natto IBRC-M 11153	

Table 3 Data obtained from the Monod equation

Batch	Specific rate of vitamin K2 formation (h^{-1})	Specific rate of glycerol consumption (h^{-1})	Vitamin K2 yield coefficient	Cell yield coefficient	μm (h^{-1})	Productivity (mg L^{-1} h^{-1})
Control fermentation medium	0.05	0.017	9.13	1.26	0.07	0.57
Batch 20% + Gly	0.1	0.013	3.64	6.22	0.06	0.12
Batch 20%	0.04	0.013	3.61	3.04	0.07	0.19
Batch 50%	0.01	0.008	6.57	2.54	0.03	0.48
Batch 75%	0.2	0.013	21.17	2.69	0.03	2.19
Batch 100%	0.02	0.013	2.2	2.11	0.05	0.44

vitamin K2 contributes to animal health similarly to humans, with deficiencies causing coagulation and skeletal issues [43].

A quantitative comparison of vitamin K2 production has been obtained from this research outcome through the employment of Monod kinetic analysis. This data provides a systematic comparison of the results due to ability of the Monod equation for relating vitamin K2 production to biomass growth and glycerol consumption. Calculations for productivity, cell yield coefficient, vitamin K2 yield coefficient, specific growth rate, specific rate of glycerol consumption, and specific rate of vitamin K2 generation are presented in Table 3. Batch 75% showed the highest specific rate of vitamin K2 formation, vitamin K2 yield coefficient, and productivity. The second vitamin K2 productivity recorded in the control medium, while batch 20% + Gly had the highest cell yield coefficient, indicating that the combination of glycerol and soapstock promoted greater biomass growth. Studies suggest that some carbon sources, like sucrose, are better for biomass conversion rather than increasing MK-7 production, as observed in 100% soapstock media [38]. The highest specific rate of glycerol consumption was found in the control medium. Catabolite repression in *Bacillus* reduces gene expression for other carbon sources when a rapidly metabolizable one, like glycerol, is present [49, 50]. The maximum value of μm was recorded in the pure fermentation medium and also in the 75% soapstock; however, the specific rate of vitamin K2 production and productivity was greater in the 75% soapstock batch compared to the control fermentation medium. From these findings, the participation of another parameter in vitamin K2 production, except μm, could be concluded. In addition, comparing the specific rate of glycerol consumption results revealed that the control fermentation medium had the greatest value, while the rates for the 20%, 50%, 75%, and 100% batches were the same. This emphasizes that the parameter leading to an increase in vitamin K2 is not related to glycerol and is possibly related to improved in situ recovery or permeability of cell membrane balance. The presence of certain fatty acids in the soapstock seems to create conditions that allow it to function as a surfactant, regulating the permeability of the bacterial membrane so that it facilitates the secretion of more extracellular vitamin K2 and might improve the recovery of vitamin K2 during the extraction process [21, 45, 51]. The productivity of batches of 50% and 100% was similar and closer to that of batch 75%, suggesting these soapstock concentrations are closer to the optimum for membrane permeability and vitamin K2 recovery. The productivity of both Gly + 20% and 20% batches was similar, as was the specific rate of vitamin K2 formation, indicating that glycerol concentration was not the primary factor affecting vitamin K2 production; otherwise, the vitamin K2 production in the Gly + 20% batch would have exceeded that of the 20% batch, indicating the effectiveness of the surfactant in vitamin K2 recovery. The vitamin K2 yield coefficient also showed a resemblance between these two batches, although glycerol had been suggested for increasing the yield of vitamin K2 production per cell [46]. When the soapstock concentration reached 50%, the vitamin K2 yield coefficient increased, implying that it could produce more vitamin K2 for the consumption of the same substrate unit. With a 75% soapstock concentration, the production of K2 reached its highest value. However, the biomass efficiency in converting the substrate to vitamin K2 was likely lowest at 100% soapstock concentration, it might be the effect of

Table 4 Comparison of vitamin K2 yield in soapstock concentration

Parameter	Control	Batch 20% + Gly	Batch 20%	Batch 50%	Batch 75%	Batch 100%
Vitamin K2 yield (mg/L)	41.58	18.57	9.35	35.7	158.16	21.18
Initial glycerol concentration (g/L)	50	50	2.6	6.5	9.75	13
Biomass (g/L)	5.25	6.51	6.33	13.8	14.4	15.2
Fermentation time (h^{-1})	72	96	144	72	72	48

Table 5 Soapstock medium parameters before and after fermentation by *B. subtilis* natto

Row	Parameter	Soapstock concentration (mg/L)	After 72h fermentation concentration (mg/L)	Difference (%)
1	COD	259,500	57,830	77.7
2	TSS	44,000	34,600	21.3
3	TDS	2920	1240	57.5
4	Nitrite	0.1	0.05	50
5	Phosphate	400	300	25
7	Total hardness	256/5	171	33.2
8	pH	10.38	7.7	25.1
9	Sulfites	3200	2600	18.7
10	Hg	–	–	0

improper cell membrane permeability balance, while a slight increase in surface tension positively affected the cell membrane's state and composition, enhancing MK-7 concentration [43].

Table 4 summarizes the vitamin K2 yield comparisons in different soapstock-containing media, along with the fermentation times spent for its production and the biomass values at those points. Result from this table indicates that the initial glycerol concentration does not have any significant effect on vitamin vitamin K2 production.

Statistical analysis of the results showed that addition of soapstock significantly affected vitamin K2 production. The groups showing the most similar results were media containing 100% soapstock, 20% soapstock + glycerol, and 20% soapstock; whereas the greatest diffrence in K2 production was found in the batch with 75% soapstock. In biomass production, the media with 20% soapstock + glycerol, 20% soapstock, and 30% soapstock were the most compareable batches and the control group was closer to the 10% soapstock medium, however the medium with 100% soapstock exhibited the greatest difference.

Effect of fermentation with *B. subtilis* natto on soapstock medium parameters

The soapstock medium was characterized by measuring the concentration of some parameters, as follows: chloride, nitrite, phosphate, ammonium, sulfite, total hardness, Hg, COD, TDS, and TSS, both before inoculation and after 72 h of fermentation (Table 5).

The COD of soapstock reduced from 259,500 mg/L to 57,830 mg/L indicating a 77% reduction. TDS diminished from 2920 mg/L to 1240 mg/L (57% reduction), and TSS dropped from 44,000 mg/L to 34,600 mg/L (21% reduction). Mercury was not detected in the culture medium with 100% soapstock before or after fermentation. In ordr to examine the effect of fermentation process on pH, this medium was not adjusted on 7 before the cultivatoin. The initial pH of soapstock was 10.38 (alkaline), which dropped to 7.7 (neutral) after 72 h of fermentation. Developing cleaner vitamin K2 production has been investigated lately, and it has been reported that there is great potential to reduce the pollution of wheat starch wastewater [48]. The result from this study could successfully imply MK-7 production and soapstock biorefining.

Conclusion

The results of study indicate that using soapstock as an alternative carbon source, instead of pure glycerol, not only did not negatively impact the fermentation process but also increased vitamin K2 production by 3.8 times with 75% soapstock. The increase is attributed to fatty acids in soapstock, which serve as a supplementary carbon source, promoting biomass growth and thereby increasing vitamin K2 production. Additionally, these fatty acids function as surfactants, regulating bacterial membrane permeability and enhancing extracellular vitamin K2 production. Also, during the fermentation process, the COD of the soapstock was reduced by 77%; consequently, this innovative use of soapstock would take the edge of the environmental pollution of soapstock industrial plants and introduce soapstock as a potential carbon source for the vitamin K2 production and in-situ recovery of it.

Abbreviations

MK	Menaquinone
HPLC	High-performance liquid chromatography
TDS	Total dissolved solid
TSS	Total suspended solids
COD	Chemical oxygen demand
ANOVA	Analysis of variance

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Author contributions

F. A. carried out the experiment and wrote the original draft of the manuscript. H.N. and H. M. edit the manuscript. The work was supervised and designed by H. N. and H.M.; and all authors read and approved the final version of the manuscript.

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Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Ethics approval is not applicable as this article does not describe any studies involving human participants or animals.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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